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Genetic diversity of the Hepatitis E virus in Switzerland

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Zusammenfassung

Die vorliegende Dissertation hat zum Ziel, die genetische Diversität des Hepatitis E Virus (HEV) in der Schweiz zu untersuchen.

Bei Schweinen im Schlachtalter resultierte eine Seroprävalenz von 59.4 %; eines von 297 getesteten Tieren war auch positiv in der RT-qPCR. Die jüngeren Tiere aus einer Kadaversammelstelle hatten eine tiefere Seroprävalenz (27.7 %), dafür wurde in 7 von 54 Schweinen virale RNA nachgewiesen. Auf einem Schweine-Bestand waren 13 von 70 Proben positiv in der RT-qPCR. Total wurden 592 Wildschweine auf HEV-Antikörper untersucht und 73 (12.3 %) davon waren positiv. Virus wurde in 7 von 143 untersuchten Wildschweine-Proben gefunden. Aus 33 in der RT-qPCR gescreenten Fleischprodukten war eines positiv. Von den HEV-positiven Fleischprodukten, konnten wir 15 von 32 erfolgreich subtypisieren. Insgesamt konnten 41 ORF-2 Sequenzen generiert werden, wovon 25 zum Subtyp 3s(p), 12 zum Subtyp 3o(p) und zwei zum Subtyp 3c (Fleisch aus Deutschland) gehörten. Eine Sequenz konnte keinem Subtyp zugeordnet werden und eine Sequenz zeigte eine Koinfektion (3o und 3s).

Zusammenfassend konnten wir bestätigen, dass in der Schweiz viele Schweine- und Wildschweinepopulationen Kontakt zu HEV haben. Dabei kommt der Subtyp 3s(p) in der Schweiz wohl am häufigsten vor und wurde bis jetzt auch nur in der Schweiz beschrieben Die Ursache für diese «genetische Insel» könnte im relativ isolierten Schweizer Schweinezucht-System liegen, welches kaum Import oder Export von lebenden Tieren sieht.

Schlüsselwörter: Hepatitis E Virus, Schwein, Wildschwein, genetische Diversität, Schweiz

Abstract

This thesis aimed at investigating the genetic diversity of Hepatitis E virus (HEV) in Switzerland.

In pigs sampled at the slaughterhouse a seroprevalence of 59.4 % resulted; one animal out of 297 was also positive in the RT-qPCR. The mostly younger animals from a carcass collection point had a lower seroprevalence (27.7 %), but in seven out of 54 pigs from this group viral RNA could be detected. On a swine farm 13 out of 70 samples tested positive in the RT-qPCR. In total, 592 wild boars were screened for HEV-antibodies and 73 (12.3 %) were positive. Viral RNA could be found in seven out of 143 examined wild boars. Out of the 33 screened meat products one tested positive. Subtyping could be achieved in 15 out of 32 additional HEV-positive meat products. Overall, 41 ORF2 sequences could be generated, whereof 25 were assigned to the proposed subtype 3s, 12 to subtype 3o(p) and two to subtype 3c (meat from Germany). One sequence could not be clearly assigned to a subtype and another revealed a coinfection (3o and 3s).

Summing up, we could confirm that HEV circulates regularly in Swiss pig and wild boar-populations. Furthermore, the subtype 3s(p) seems to be predominant in Switzerland and, to date, has only been detected in Switzerland. The reason for this “genetic island” may lie in the relative isolation of the Swiss pig industry, where hardly any animals are imported or exported.

Keywords: Hepatitis E Virus, pig, wild boar, genetic diversity, Switzerland

Virus Glossary

APPV	Atypical Porcine Pestivirus
BVDV	Bovine Viral Diarrhoea Virus
HEV	Hepatitis E Virus
PCV	Porcine Circovirus
TTV	Torque Teno Virus

1. Introduction

1.1 The virus

1.1.1 Short history – Discovery of Hepatitis E virus

The Hepatitis E virus was first discovered in a group of Soviet soldiers posted in Afghanistan in 1983, who suffered from an unexplained hepatitis. The Soviet virologist Dr. Balayan infected himself orally with a pooled stool sample from these supposed cases of epidemic non-A, non-B hepatitis. Under the electron microscope he detected spherical viral-like particles in stool samples collected in the clinical phase (Balayan et al., 1983). In 1990 the virus was renamed from 'enteric non-A non-B hepatitis' (ENANBH) to Hepatitis E virus (HEV) (Reyes et al., 1990).

1.1.2 The particle and its genome

With 32-34 nm in diameter the HEV particle is relatively small. The fact that it is a so called quasi-enveloped virus is remarkable and the first hint towards this feature of the virus was discovered by Takahashi et al. (Takahashi et al., 2008). In serum and faeces no envelope encloses the particle whereas in blood and cell-culture a lipid derived membrane forms a capsid. The unenveloped particle goes along with a high infectivity and the enveloped with a lower infectivity (Nimgaonkar et al., 2018). The quasi-envelope hides the virion from neutralizing antibodies in the blood stream, but lowers the efficiency of the virus to enter cells (Yin et al., 2016).

A single stranded, positive-sensed RNA is embedded in the HEV-virion. It is 7.2 kb long and consists of three open reading frames (ORF) (Tam et al., 1991). The information for the RNA-dependent RNA-polymerase (RdRp) and other non-structural proteins are included in the longest ORF, the ORF1 (Fry et al., 1992). The ORF2-region encodes for the major capsid protein (Jameel et al., 1996) and the smaller ORF3 is important for the release of the HEV-particles. Only some years ago it was shown that this 360 bp long genome region encodes a membrane ion channel (Ding et al., 2017).

1.1.3 Classification

The HEV belongs to the family of the *Hepeviridae*. This family is composed of the genera *Orthohepevirus* and *Piscihepevirus*. All avian and mammalian isolates of the virus are assigned to the first, the latter consists of the cutthroat trout virus (Smith et al., 2014). Subsequently, we will focus on the species *Orthohepevirus A* in the first above mentioned genus, as the viruses of interest for this study are classified there. The species *Orthohepevirus A* contains at least eight genotypes. Genotype 1 and 2 are limited to humans. Genotype 3 and 4 are zoonotic and infect humans, the main reservoir host is the pig, but also several other species like wild boars and deer. Genotype 5 and 6 are restricted to wild boars and genotypes 7 and 8 infect camels (Meng, 2016; Smith et al., 2014). Figure 1 shows all the eight genotypes with their hosts, whether they infect humans and the ways of transmission to them.

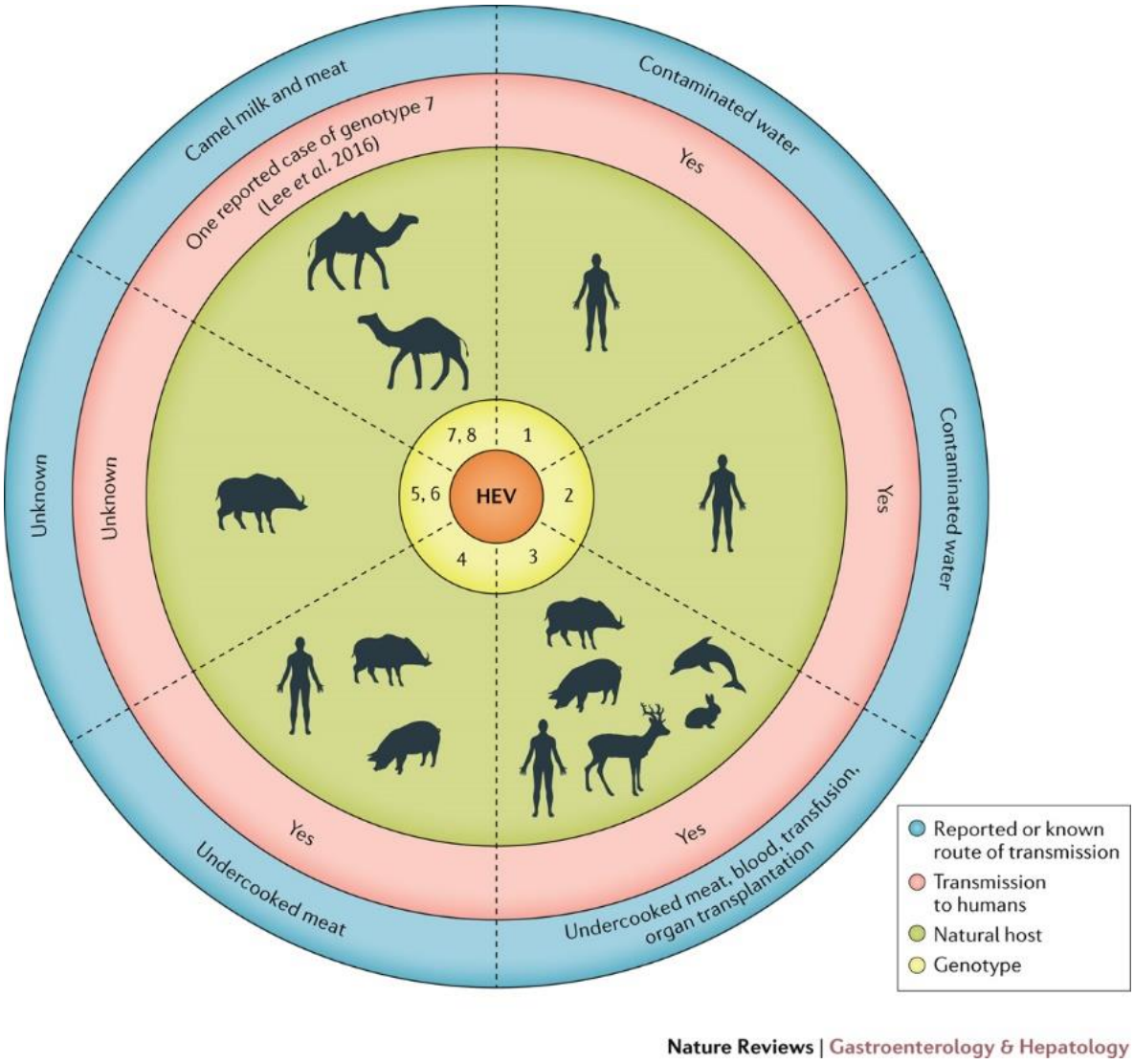

Figure 1: Host range and routes of transmission to humans of HEV genotypes 1-8 (Nimgaonkar et al., 2018).

1.1.4 Epidemiology

HEV is spread worldwide. Especially genotypes 1 and 2 are a major health issue in developing countries where drinking water may be contaminated with the virus. In 2015 around 44`000 deaths were attributed to this disease according to the WHO (https://www.who.int/en/news-room/fact-sheets/detail/hepatitis-e). Genotype 1 is endemic in Africa and Asia, genotype 2 was isolated in Mexico (Huang et al., 1992) and in different African countries like Nigeria (Buisson et al., 2000). Genotype 3 is present worldwide, but of major importance in industrialized countries. In Europe it is the most frequently detected genotype. A high variety of subtypes is already described for this genotype (Izopet et al., 2019). Genotype 4 is mostly present in Asian countries like China and Japan (Lapa et al., 2015), yet single cases of autochthonous infections were also reported in Europe, for example in Germany (Wichmann et al., 2008) and Switzerland (Fraga et al., 2017). The genotypes 5 and 6 have only been detected in wild boars in Japan (Li et al., 2015). Finally, number 7 and 8 are present in dromedary

and camels, but also in humans (gt7) and monkeys (gt8) (Lee et al., 2016; Wang et al., 2019).

1.2 The HEVnet database and the publicly available HEV typing tool

The HEVnet network (https://www.rivm.nl/en/hevnet) was initiated by the European Centre for Disease Prevention and Control (ECDC) and established in April 2017 by the Dutch National Institute for Public Health and the Environment (RIVM) in order to share molecular and epidemiological data on HEV globally. The heart piece is a database where HEVnet members working in the medical, public health, animal health or blood donation sector have a login and can submit HEV sequences. This platform secures standardized genotype and subtype assignment as proposed by Smith et al. (Mulder et al., 2019; Smith et al., 2016). The aim is to work in a one health setting and bring together metadata (source, place, time, clinical information) to be able to learn more about circulating HEV strains in Europe and the epidemiology of the virus (Mulder et al., 2019). The Institute of Virology of the University of Zurich is also member of HEVnet and sequences produced in this thesis were submitted to the HEVnet database.

The second important tool is a publicly available online geno- and subtyping platform, the Hepatitis E virus genotyping tool, that can be used by anyone to blast their HEV sequences. It facilitates a uniform HEV taxonomy and nomenclature. The sequences uploaded by HEVnet members to the password protected database are automatically typed by this tool as well (https://www.rivm.nl/mpf/typingtool/hev/).

1.3 HEV genotype 3 and its epidemiology in Europe

We will focus mainly on genotype 3, as this is the relevant genotype for this study. HEV genotype 3 was first discovered in a human case in the USA (Kwo et al., 1997). As HEV was mainly known as a travel-related disease in industrialised countries, only some years later divergent data was published. In 2004 Mansuy et al. identified HEV-3 strains circulating in France, infecting patients without any recent travel history (Mansuy et al., 2004). Another study following the same hints proved, that autochthonous HEV infections in Europe are far more common than previously known and any unexplained hepatitis should be tested for this pathogen (Dalton et al., 2008; Mansuy et al., 2004). From 2005-2015 a total of 20´000 HEV cases, all genotypes included, were reported in Europe and 97 % of those were locally acquired (Adlhoch et al., 2019).

In European countries HEV is mostly transmitted via the consumption of undercooked or raw pork and wild boar liver products or meat (Lapa et al., 2015). In his review Pavio et al. summarized the main routes of zoonotic HEV-transmission and draws the same conclusion: The consumption of meat products (pork liver, pork products containing liver, other meat consumed raw or undercooked) reigns supreme on top of all the routes of zoonotic HEV infection (Pavio et al., 2017). The *Figatellu*, a traditional sausage from France containing raw pork liver, is a very good example for a high-risk product concerning HEV. It was even proven that the virus inside this sausage was still

infectious by using cell culture (Berto et al., 2013a; Berto et al., 2013b). In Switzerland different types of sausages, such as *Mortadella cruda* or *Lebersalsiz*, local specialities in the cantons Ticino and Grisons, contained HEV RNA. All sequences detected were assigned to genotype HEV-3. In this study none of the 15 products containing game meat was tested positive (Moor et al., 2018).

One of the best reported cluster outbreaks concerning game meat originates from Asia. After several people got ill upon consumption of raw deer meat in Japan, Tei et al. showed that the meat was positive for HEV RNA and the sequence identical to the one from the patients (Tei et al., 2003). HEV antibodies and RNA were also detected in deer populations in European countries, such as Germany and Italy. Neumann et al. proved the presence of the virus in German red, roe and fallow deer populations (Neumann et al., 2016). In Italy the virus is prevalent in the red deer population and two fragments of the genome which could be sequenced matched with HEV-3 genotype (Di Bartolo et al., 2017).

The direct (work-related) contact to pigs, wild boars or deer is another transmission route of zoonotic HEV and identified as a risk factor for different groups, such as pig farm workers, slaughterers or swine veterinarians (Bouwknegt et al., 2008; Chaussade et al., 2013; Krumbholz et al., 2012). Other potential transmission routes are blood transfusions (Hewitt et al., 2014), shellfish consumption (Crossan et al., 2012) or ingestion of leafy green vegetables that are contaminated with the virus (Kokkinos et al., 2012).

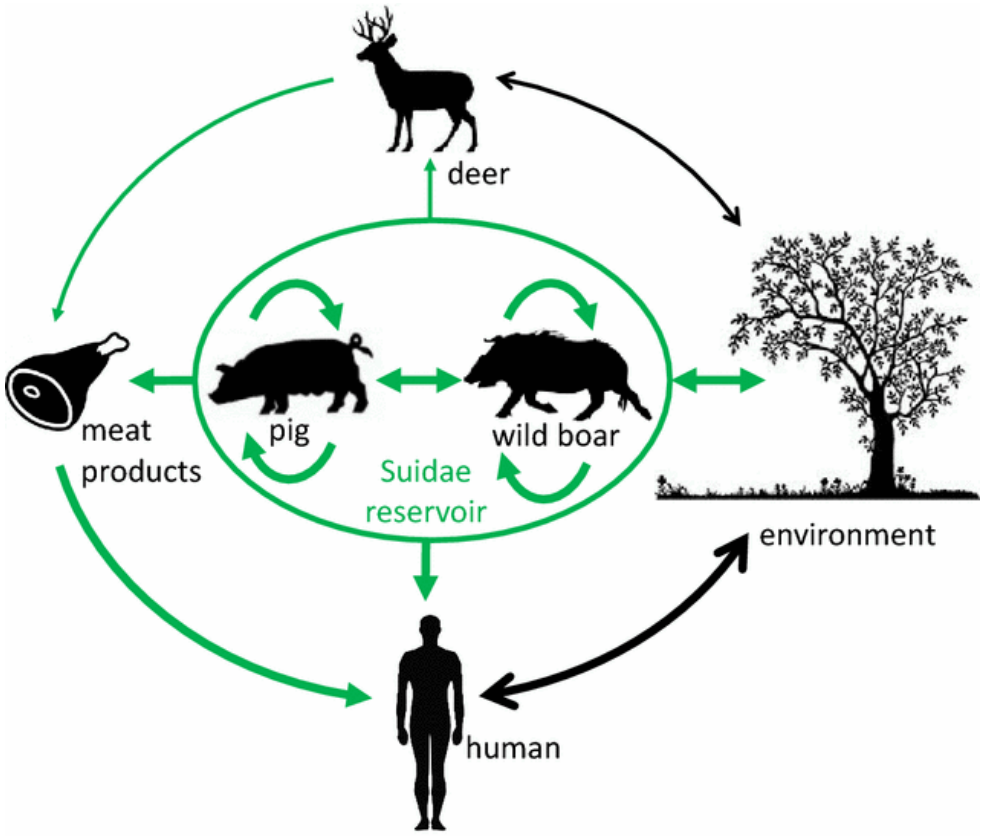

Figure 2: Routes of transmission for zoonotic HEV. Green arrows show proven ways of infection and the thickness of the arrows indicates the importance of the respective route (Pavio et al., 2017).

HEV genotypes can be further divided into subtypes. These are genetically related but cluster without a clear demarcation cut-off. A growing number of subtypes of HEV-3 have been sequenced in the last few years. In the following we will have a closer look at countries bordering Switzerland, concerning the HEV-3 subtypes circulating there. The subtypes 3c and 3f are predominant in France according to the online HEVnet sequence database. This is confirmed by the findings of Izopet et al (Izopet et al., 2019). Other subtypes that are present in France, but less often submitted to the online database, are 3h, 3l(p), 3r(p). The strains from Germany which were submitted to the HEVnet database belong to subtype 3c and 3q(p). Several studies showed also the presence of subtypes 3a, 3c, 3e, 3f, 3h and 3i in this country (Adlhoch et al., 2009; Anheyer-Behmenburg et al., 2017; Beyer et al., 2020; Weigand et al., 2018; Wenzel et al., 2011). In Italy, again 3c and 3f are the main subtypes reported to the HEVnet database, but also many other subtypes with Italian origin were uploaded there (3a, 3e, 3i, 3l(p), 3o(p), 3t(p), 3w(p)). This finding of a big variety of subtypes being present in Italy, is also reflected by different published studies (De Sabato et al., 2020; De Sabato et al., 2018; Di Pasquale et al., 2019; Iaconelli et al., 2020; La Rosa et al., 2017). For Austria, no sequences were reported to the HEVnet database up to date. Studies have shown that genotype 3 is present, however no subtypes have been reported so far (Zwettler et al., 2012).

1.4 HEV gt3 in pigs (Sus scrofa domesticus), wild boars (Sus scrofa scrofa) and other reservoir hosts

Pigs, wild boars and deer are true hosts of HEV and are therefore important sources of infection for humans (Van der Poel, 2014). Meng et al. isolated and characterized the first animal strain of HEV from piglets in the United States (Meng et al., 1997). Pigs do not exhibit any clinical signs, only microscopical lesions can be found in the liver of infected animals (Halbur et al., 2001). On the single animal level the virus is transmitted faecal-orally (Kasorndorkbua et al., 2004), which can induce an accumulation of HEV in the environment of the pigs at the different production stages and in the manure pits (Fernández-Barredo et al., 2006; Salines et al., 2017). Different tissue samples and excreta, such as liver, muscle tissue, lymph nodes, pancreas, kidney, faeces and urine have been identified to contain HEV RNA in swine (Bouwknegt et al., 2009). As mentioned, in wild boar and deer the virus was shown to be present in muscular tissue and liver (Takahashi and Okamoto, 2014; Tei et al., 2003), however the exact organ distribution is less well analysed in these species than in pigs.

The course of the HEV infection in the domestic pig is documented in various studies and many divergent findings are reported. Shortly, the faeco-oral infection is supposed to happen between one up to three months of age. The exact spread of the virus in the body remains to be elucidated, but one verified site of replication is the liver. The viral particles are released to the bile and the blood. The viremia goes on for one to two weeks and the subsequent faecal shedding can last from two weeks up to one month (Fernández-Barredo et al., 2006; Salines et al., 2017; Schlosser et al., 2014; Seminati et al., 2008). Excretion via the urine has been proven as well (Bouwknegt et al., 2009) Three months old pigs seem to be the major shedders on farms (Salines et al., 2017).

Seroconversion to HEV is reported to occur between 3 and 8 weeks post infection, but these numbers have to be handled with care as they vary from publication to publication (Kasorndorkbua et al., 2002; Meng et al., 1998; Schlosser et al., 2014). At the timepoint of slaughter, HEV RNA is still detectable in pig livers as studies from different countries show. However, the range of the percentage of positive livers at slaughtering age is wide, going from 4 % in France up to 20.8 % in Italy (Di Bartolo et al., 2011; Rose et al., 2011).

Wild boars (*Sus scrofa scrofa*) are another reservoir for HEV in Europe. In different countries the seroprevalence in the wild boar population has been studied, for example in Italy (40.7 %) (Montagnaro et al., 2015), Germany (11.5 %) (Weigand et al., 2018), France (14 %) (Carpentier et al., 2012) or Switzerland (12.5 %) (Burri et al., 2014). Schlosser showed, that horizontal transmission of HEV from wild boars to domestic pigs is possible (Schlosser et al., 2014). Another study supported these findings, as the authors reported a case of a chronically infected wild boar which transmitted a genotype 3 virus strain to domestic sentinel pigs (Schlosser et al., 2015).

In roe deer (*Capreolus capreolus*) the seroprevalence seems to be lower than in wild boars, for example in a study from Germany it ranged from 5.4-6.8 % (Neumann et al., 2016). In another German study HEV RNA was detected in the liver and muscle tissue of roe deer samples (Anheyer-Behmenburg et al., 2017).

Another deer species being relevant as a reservoir host for HEV in Europe is the red deer (*Cervus elaphus*). Viral RNA and antibodies against HEV were detected in red deer populations in Germany (Anheyer-Behmenburg et al., 2017; Neumann et al., 2016) and also Italy (Di Bartolo et al., 2017). However, percentage of HEV positive red deer was relatively low in these studies from Europe, sometimes also lower than the prevalence in roe deer. In a German study following the red and roe deer population over three hunting seasons, 6.4 % of the roe deer and 2.4 % of the red deer population was tested positive for HEV RNA (Anheyer-Behmenburg et al., 2017).

HEV strains isolated from pigs, wild boar and deer belong predominantly to genotype 3 (Di Bartolo et al., 2011; Montagnaro et al., 2015; Schielke et al., 2009). Single genotype 4 strains have been found in pigs in Europe as well, for example in Italy where 57 pig farms were sampled. Most of the HEV strains detected belonged to genotype 3, but also genotype 4 was sequenced (Monne et al., 2015).

A zoonotic rabbit HEV-strain (3ra) exists as well and has also been detected in humans in Switzerland (Sahli et al., 2019). Remarkably, none of the patients in this Swiss study being infected with rabbit HEV has had contact with rabbits or consumed rabbit meat, which is in line with earlier findings and leads to the conclusion, that this virus must have found other ways to infect humans (Abravanel et al., 2017).

1.5 HEV gt3 in humans

In healthy adults an infection with Hepatitis E virus is often self-limiting and asymptomatic. However, in immunosuppressed patients or people with an underlying liver disease severe courses of the disease, like an acute hepatitis or a chronic

infection, are possible, the latter especially in solid organ-transplant recipients (Lhomme et al., 2016). Extrahepatic manifestations are frequent and include neurological symptoms like the Guillain-Barrée syndrome (Tse et al., 2012) and neuralgic amyotrophy. An impaired renal function due to HEV-infection has been described as well (Kamar et al., 2015).

The seroprevalence in Europe among blood donors shows a variety from a relatively low percentage of 4.7 % in Scotland, up to more than 50 % in the southwestern part of France (Cleland et al., 2013; Mansuy et al., 2011).

In a study from 2019 17 out of 30 European countries had established a national surveillance program for HEV (Adlhoch et al., 2019). Blood donors are routinely tested on HEV RNA in several European countries, such as Switzerland, the United Kingdom, Germany and the Netherlands (Denner et al., 2019; Domanović et al., 2017; Niederhauser et al., 2018). Considering the fact that patients receiving blood donations are often immunosuppressed or severely ill otherwise, it is stated by some researchers that all blood donations should be tested on HEV (Denner et al., 2019).

1.6 HEV in Switzerland

In Switzerland the seroprevalence of HEV in domestic pigs at slaughter was as high as 58.1 % in 2014. The same study showed that 12.5 % of the Swiss wild boar population was positive for anti-HEV antibodies (Burri et al., 2014). The prevalence of HEV in 160 pig livers of 40 different Swiss fattening farms was examined in 2017 and determined to be 1.3 % (Müller et al., 2017). In the human population 20.4 % of blood donors were tested positive on anti-HEV antibodies (Niederhauser et al., 2018). Other publications revealed the presence of HEV RNA in liver sausages and meat products in Switzerland, such as the liver sausage *Mortadella di fegato,* a specialty from the canton Ticino (Giannini et al., 2018; Moor et al., 2018).

Since January 1st in 2018 Hepatitis E is a reportable disease in Switzerland. In 2018 a total of 20 cases was reported, in 2019 already 44 cases were reported and on the 11th of May the number for 2020 has already reached 32 cases (https://www.bag.admin.ch/bag/de/home/das-bag/publikationen/periodika/bag-bulletin.html).

Concerning the strains circulating in Switzerland, Fraga et al. showed in 2017 that mainly HEV genotype 3 strains are present in humans with clinical manifestation, but also single genotype 4 infections acquired in Switzerland were detected (Fraga et al., 2017). In the same year, a full-length HEV genome was isolated from a kidney transplant recipient in Switzerland. Phylogenetically this strain was classified as a genotype 3, but it shared less than 88 % of the sequence with published strains and therefore the authors postulated that they might have discovered a new HEV-3 subtype (Wang et al., 2017). Shortly after, our lab published an interesting case concerning a stool sample from a 78-year old male patient and the *Mortadella di fegato crudo* he had eaten. From both samples the identical full length HEV-genomes could be sequenced and again it seemed, that they belong to this new probably Swiss-specific

cluster of HEV-3 viruses (Kubacki et al., 2017). Going deeper into the topic, a master thesis at the Institute of Food Safety and Hygiene at the Vetsuisse Faculty in Zurich revealed, that HEV sequences from Swiss fattening pigs belonged to the same cluster, the proposed HEV-3s(p) subtype (Wist et al., 2018). It is important to note, that the subtype 3s was named by the people responsible for maintenance and support of the HEVnet typing tool, which provides preliminary names to newly reported subtypes alphabetically. Therefore the “s” does not stand for “Swiss”, which could be assumed without having the context and the “s” is followed by a “(p)” standing for “proposed”.

1.7 Aims

The aim of this dissertation thesis was to investigate the genetic diversity of HEV along the food chain in Switzerland in an explorative study. The question of a potential “Swiss” HEV-3 subtype (proposed 3s) has recently aroused and more investigations are necessary to shed light on the question, whether Switzerland may be an island concerning the genetic diversity of this virus, if in fact a “Swiss” subtype exists and if so, what could be the reasons for this genetic “outlier”. Another claim of this study is to get to know the range of HEV-strains circulating in the swine and wild boar population in Switzerland and gain more information on this relevant zoonotic disease from the viewpoint of the veterinary medicine. More background on the spread of the virus in different animal reservoir hosts in Switzerland is necessary to follow infections from farm to table and to trace chains of infections in humans. We need to know where and why humans get infected in our country to be able to break up these infection cycles. The presence of HEV in autochthonous human cases in Switzerland has underlined the relevance of being able to trace back human cases (Fraga et al., 2017).

2. Materials and methods

2.1 Animals and sample material

For the purpose of exploring the genetic diversity of HEV in Switzerland different types of samples and animal species were tested. All samples were stored at -20°C until testing. Figure 3 displays the approach applied on all samples in this study.

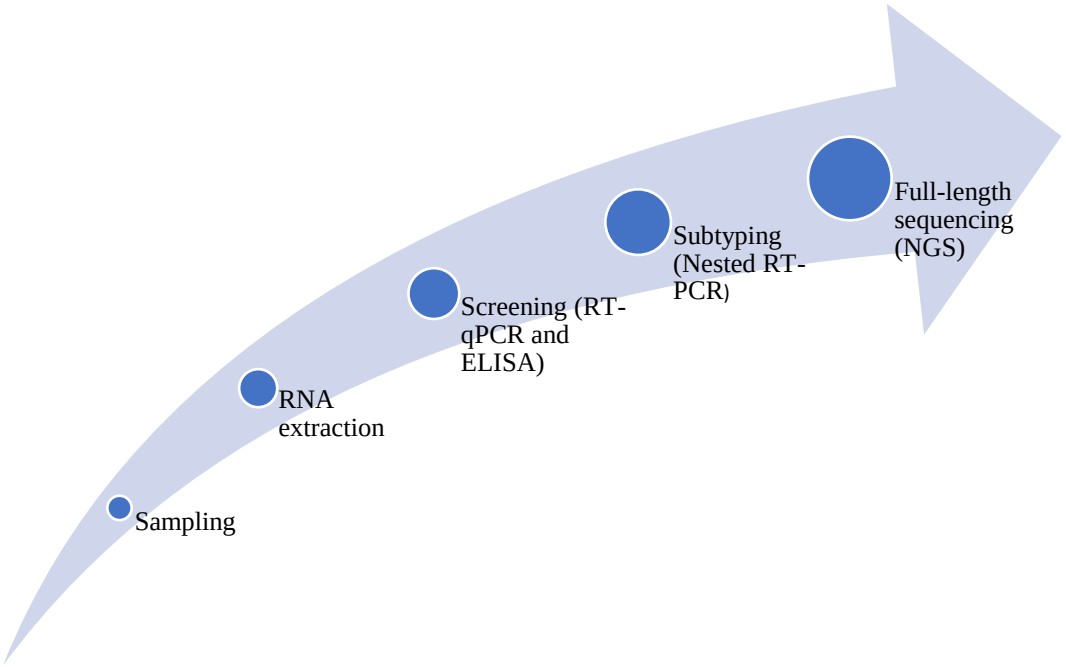

Figure 3: Approach used in this study to deal with (potentially) HEV-positive samples.

Table 1 gives an overview of all the samples originating from animals tested in this study, which included pigs, wild boars and roe deer.

Table 1: Overview of all samples originating from animals tested in this study.

Number of samples	Species	Sample Material	Origin	Provided by
297	Fattening pigs	Liver	Northern/Western Switzerland	Slaughterhouses Zurich, Basel, Courtepin
162	Pigs of all age categories	Liver, diaphragm, faeces	Canton of Lucerne, Switzerland	Prof. Xaver Sidler, Swine clinic, Vetsuisse Faculty Zurich
70	Rearing and fattening pigs	Floor/dust swabs, liquid manure, faeces	Canton of Aargau, Switzerland	Swine clinic, Vetsuisse Faculty Zurich
125	Wild boar	Liver (Austria: Liver, diaphragm, faeces)	Canton of Schaffhausen and Ticino; Austria	Prof. Cornel Fraefel and huntsmen, Veterinary office of canton of Ticino and huntsmen, Prof. X. Sidler
467	Wild boar	Diaphragm	Cantons of Schaffhausen, Aargau, Zurich, Basel	Veterinary offices of the canton Grisons (Samples from Basel are tested here as well), and Schaffhausen; Institute of Parasitology University of Zurich (Samples from Aargau are tested here as well)
14	Roe deer	Liver	Canton of Schaffhausen, Switzerland	Prof. Cornel Fraefel and huntsmen from Schaffhausen
1	Deer	Muscle Tissue	Germany	Karin Dietze

2.1.1 Pigs

Liver samples from pigs at the timepoint of slaughter, which is normally at six months of age, were collected between May and June 2018 in three big slaughterhouses in Switzerland. Of the total yearly amount of pigs slaughtered around 50 % are processed in Zurich, Courtepin and Basel (Personal communication Prof. Roger Stephan, Director Institute for Food Safety and Hygiene, University of Zurich). In Zurich, 74 animals were sampled, in Basel 58 and from Courtepin 60 livers of healthy animals and 105 confiscated liver samples were gathered by slaughterhouse staff members for this work. Confiscated livers are assigned as not being fit for human consumption, e.g. due to macroscopic lesions, for example due to parasitological infections in the liver. Since the pigs shed the Hepatitis E virus mainly around three months of age also younger pigs were of interest for the analysis and therefore 54 animals were sampled by Prof. X. Sidler in two cadaver collection points (CCP) in the canton Lucerne, more exactly in Hochdorf (n=24) and Knutwil (n=30), in March and August 2018. The canton Lucerne is the main pig breeding and fattening area in Switzerland (Burri et al., 2014). As sample material, a part of the liver, the diaphragm and faeces were collected from each animal individually. Additionally, pig samples collected on one pig farm, called FU pig farm, by the Swine clinic of the Vetsuisse Faculty in Zurich were tested for the virus. They had been collected for a doctoral thesis in 2017. Basically, single animals were followed on this farm as they grew up and sampled at two timepoints. The first timepoint was K1, when the pigs were about ten weeks old and still in the rearing unit. On timepoint two (K3) about two to three weeks later the same animals were sampled a second time. From the pig pens where the pigs in question lived in, environmental

samples such as swab samples from the pen floor, mixed faecal samples, manure and dust samples were gathered at the same timepoints. For the detailed number of samples see table 2. The floor swabs analysed in this study, consisted of a gauze sponge moistened with sterile saline and wiped on the floor and walls of the pig pen where the corresponding pigs of the study were housed in. From horizontal surfaces near the pens (e.g. windowsills, water pipelines) a dust swab was collected in the same procedure. The liquid manure samples were collected from the closest slurry pit.

Table 2: Number of samples from the FU pig farm tested on HEV originating from a doctoral thesis in the Swine clinic of the Vetsuisse Faculty in Zurich dating from 2017.

Sample type	**Timepoint of sampling**	
	K1 (Rearing)	K3 (Fattening, day 14-21)
Floor swab	3	4
Dust swab	3	4
Liquid manure	3	4
Faecal sample single animal	29	20

2.1.2 Wild boars

From December 2017 to March 2019 a total of 75 liver samples was collected by hunters in the canton Schaffhausen. Another 46 liver samples originate from the canton Ticino. These animals were shot in September 2018. Four wild boars hunted in May 2018 in the Burgenland in Austria were sampled as well. In this last case we received liver, diaphragm tissue and a faecal sample from each individual animal.

All wild boars hunted in Switzerland need to be tested on the zoonotic parasite *Trichinella* and therefore muscle tissue samples, normally from the diaphragm, are sent to the responsible laboratory by the hunters. What is left after testing is stored at -20°C for a couple of weeks and then discarded. Instead of throwing these samples away they were sent to our laboratory by the responsible department to be tested on HEV antibodies. The samples from the cantons Zurich and Aargau were provided by the Institute of Parasitology of the University of Zurich, the samples from Schaffhausen by the Cantonal Veterinary Department Schaffhausen, the ones from Basel and Solothurn by Dr. Paulin Zumthor from the Department of Food Safety and Animal Health of the canton Grisons, as they test the *Trichinella* samples for Basel/Solothurn as well. In total 141 diaphragm samples from the canton Schaffhausen, 87 from Aargau, 64 from Zurich, 92 from Basel-Land, and six from Solothurn were gathered from November 2018 up to June 2019. For part of the diaphragm samples (n=55) no information regarding the origin of the samples was available and 22 samples originated from outside Switzerland (France, Germany, Czech Republic).

2.1.3 Deer

Prof. C. Fraefel and other hunters from the canton Schaffhausen additionally collected 14 roe deer liver samples for the present work to be tested on Hepatitis E antibodies and virus.

2.1.4 Meat products

The Federal Food Safety and Veterinary Office (BLV) in Berne provided a total of 21 food samples that were tested positive for HEV in their own lab between March 2016 and May 2018. From the Cantonal Laboratory of the canton Ticino, eleven HEV-positive *Mortadella*-sausages were supplied to our lab for genetic analysis of the virus. These samples were taken between August 2015 and June 2017. The company Bell Food Group AG sent us 20 different pork and game meat products by their own choice to test for HEV in October 2018. In addition, 13 different sausages and meat products which we acquired privately were included in this study. An overview of the food samples is given in table 3.

Table 3: Food samples tested in this study.

Number	Meat product	Origin	Provided by
21	Different kinds of (raw) sausages and different pork meat products	Switzerland, Germany	Dominik Moor, BLV Berne
11	*Mortadella*	Canton of Ticino	Cantonal Laboratory of the canton Ticino
20	Different kinds of sausages, pork and game meat products	Switzerland, Hungary, Austria, Slovenia	Bell Food Group
13	Different kinds of raw sausages and pork and wild boar meat products	Switzerland, France, Poland, Spain, Germany	Privately acquired

2.2 RNA extraction

The QIAgen Viral RNA Mini Kit (Qiagen, Switzerland) was used to extract the RNA from most of the samples. After the material-specific sample preparation, described below in detail, the kit was performed according to the manufacturer's instructions, using 140 µl input volume. For the elution of the RNA 50 µl nuclease-free water was used. The exception of this process were the highly processed food samples, which were extracted chemically using a Trizol-based method.

2.2.1 Sample preparation: Liver samples

From the frozen liver tissue 30 mg were weighed in a 2 ml safe-lock Eppendorf tube. In a next step 200 µl of nuclease-free water and a 5 mm steel bead (Qiagen, Switzerland) were added to the tube. Samples were then homogenized for 30 seconds at 25 Hz in the Tissue Lyzer II (Qiagen, Switzerland). After a three-minute centrifugation step at 13`000 rpm (app. 16`000 g), the supernatant was used in the QIAgen Viral RNA Mini Kit.

2.2.2 Sample preparation: Faecal samples and liquid manure

For the RNA extraction from the faecal samples/liquid manure 100 mg of faeces or liquid manure were weighed in a 2 ml Eppendorf tube and the 10-fold volume of PBS added to the tube. Samples were then homogenized for 30 seconds at 25 Hz in the Tissue Lyzer II (Qiagen, Switzerland). After a five-minute centrifugation step at 13`000 rpm (app. 16`000 g) the sample was ready to be extracted.

2.2.3 Sample preparation: Floor and dust swabs

To be able to extract the nucleic acid the swab was defrosted at room temperature inside the plastic bag in which it was stored, and the gauze manually kneaded to extract the fluid. Approximately 500 µl fluid were transferred to a 2 ml tube, vortexed for at least 15 seconds and then centrifuged for 1 min full speed. With 140 µl of the resulting supernatant the extraction was accomplished.

2.3 RNA extraction from food samples

Highly processed food samples, such as very dry and/or firm sausages like *Salsiz*, were extracted chemically using TRIzol LS Reagent (Thermo Fisher Scientific, Switzerland). The protocol provided by the producer was adapted as described by Moor et al. and based on own experiences (Moor et al., 2018). For details see 2.3.2.

2.3.1 Sample homogenisation of sausages and meat products

Very firm and dry sausages were rather challenging to homogenise. Therefore, a three-step homogenisation approach was used. Of each sausage 500 mg was weighed and added to 500 µl water in a 2 ml tube to be soaked/pre-homogenised without bead by running the Tissue Lyzer for 1 min at 25 Hz. In a next step a 5 mm steel bead was added and again the tube was put in the Tissue Lyzer for 1min/25Hz. Of the resulting squish 100 mg was transferred into a new tube, 200 µl of water and another steel bead were added. One last time the Tissue Lyzer was operated for 1min/25Hz. From the softer sausages, 200 mg were weighed to a tube containing 700 µl of PBS and a 5 mm steel bead. Homogenisation was completed by running the Tissue Lyzer for 1 min at 25 Hz. If necessary (e.g. still big lumps in the tube), the last step was repeated. As a last step of the sample preparation the samples were centrifuged for 3 min at 13`000 rpm.

2.3.2 RNA extraction from sausages and meat products

The resulting supernatant was transferred to a new tube. In order to avoid transferring fat, the pipette tip was cleaned with a paper towel before adding the liquid to the new tube. Normally, a minimum of 200 µl up to a maximum of 350 µl of supernatant could be gained. The volumes used in this extraction protocol are shown in table 4; they were adapted relative to the volume of the supernatant used. As a first step the TRIzol LS Reagent was added and the sample was mixed by pipetting up and down several times. After an incubation step of at least 5 min at room temperature the chloroform was added, and the sample incubated for 3-10 min at room temperature. Next, the sample was centrifuged for 5 min at 12`000 g and 4°C for phase separation. The clear aqueous phase, containing the RNA, was transferred to a new tube and glycogen was added. After addition of the isopropanol and mixing by pipetting up and down carefully, the sample was incubated at 4°C for at least 15 min. Another centrifugation step followed (15 min at 12`000 g and 4°C) to pellet the RNA. The supernatant was removed, and 1 ml 75 % Ethanol added, to wash the pellet. The tubes were spun for 10 min at 12`000 g at 4°C and the washing step was repeated a second time. All the

ethanol was carefully removed, and the pellet air-dried. Resuspension of the pellet was accomplished with 100 µl of DEPC water.

Table 4: Volumes used in the TRIzol extraction for food samples.

Reagent	Volume	Relative
Sample	200-350 µl	1
TRIzol LS	600-1050 µl	3
Glycogen (20 µg/µl)	1.14-2 µl	0.0125
Chloroform	160-280 µl	0.8
Isopropanol	400-700 µl	2

2.4 RT-qPCR screening

Quantitative reverse-transcription PCR (RT-qPCR) was performed on a Quant Studio 7 or Quant Studio 3 Real Time PCR System (Applied Biosystems, USA). The protocol for the RT-qPCR had been previously established (Jothikumar et al., 2006) and was adapted for our lab. Differing from the published protocol, a TaqMan MGB probe was used (Adaptation by Dr.Samreen Ijaz, National Institute for Health Research, London, UK). This RT-qPCR is suited to detect genotype 1-4 of Hepatitis E virus. As a PCR reagent, the QuantiTect Probe RT-PCR Kit (Qiagen, Switzerland) was used until November 2019 when it was not manufactured anymore. The new kit proposed by Qiagen to substitute the QuantiTect was the QuantiNova Pathogen +IC Kit (Qiagen, Switzerland). To be sure that the QuantiNova kit worked for our purpose as well, we tested these two kits with the same samples in parallel. As the performance was comparable and we got basically the same CT values the new kit was established. Primers and probe are listed in table 5. Reagents of the QuaniTect Probe RT-PCR kit were mixed according to table 6 with a final reaction volume of 20 µl whereof 8 µl was RNA. The total volume for the reaction mix with the QuantiNova kit was set to 8 µl and 2 µl of RNA (Tab. 7). In this kit, an internal control is included, which can be used as extraction or inhibition control. The cycling conditions of the two kits can be found in tables 8 and 9. In case of a positive result, the RNA extraction of the same sample was repeated and tested again, to confirm the result.

Table 5: Primer and probe sequences for HEV RT-qPCR, reference strain M73218 (Jothikumar et al., 2006).

Primer	Sequence	Position
Forward (JVHEV_F)	5´-GGTGGTTTCTGGGGTGAC	5261-5278
Reverse (JVHEV_R)	5´-TTCATCCAACCAACCCCT	5313-5330
Probe (JVHEV_P)	5´-FAM-TGATTCTCAGCCCTTCGC-MGB-3´	5284-5301

Table 6: Reaction setup QuantiTect RT-qPCR.

Reagent	Volume (µl)	Concentration
5x NR Master Mix	4	1x
QuantiTect Virus RT Mix	0.2	1x
JVHEV_F	0.8	0.4 µm
JVHEV_R	0.8	0.4 µm
JVHEV_P	0.4	0.2 µm
ROX	0.4	50 nm
Water	5.4	
RNA	8	Variable
Final volume	**20**	

Table 7: Reaction setup QuantiNova RT-qPCR.

Reagent	Volume (µl)	Concentration
4x QuantiNova Master Mix	2.5	1x
HEV primer/probe mix	0.5	0.8 µm/0.8 µm/0.25 µm
QN IC probe assay VIC	1	
QN ROX	0.05	
Water	3.95	
RNA	2	Variable
Final volume	**10**	

Table 8: Cycling conditions with the QuantiTect kit.

C°	Duration	Cycles
50	20 min	
95	5 min	
95	15 sec	45
60	45 sec	

Table 9: Cycling conditions with the QuantiNova Pathogen +IC Kit.

C°	Duration	Cycles
50	10 min	
95	2 min	
95	5 sec	45
60	45 sec	

2.5 RNA extraction control

2.5.1 Detection of porcine and bovine 12S

To control for successful RNA extraction from porcine and ruminant tissue, housekeeping genes, the porcine (p12S) and ruminant mitochondrial 12S rRNA coding genes, were measured by real-time RT-PCRs used by the diagnostic unit of the Institute of Virology. The p12S RT-PCR is specific for pigs and was therefore used for all porcine and wild boar samples. As we always had very strong signals for p12S (hence very low CT values), the RNA was pre-diluted 100-fold with PBS. For the deer samples the bovine 12S coding gene was used, which is specific for ruminants. The housekeeping gene RT-PCRs were run with the QuantiTect Kit and the same conditions as described above for HEV. The only difference was that only 1 µl of RNA was used instead of 8 µl. The following primers and probes, displayed in table 10 and 11, were used for the detection of the housekeeping genes p12S and ruminant 12S:

Table 10: Primer and probe sequences for detection of porcine housekeeping gene p12S.

Primer	Sequence
Forward (p12S_F)	5'-CCACCTAGAGGAGCCTGTTCTATAA-3´
Reverse (p12S_R)	5'-GGCGGTATATAGGCTGAATTGG-3´
Probe (p12S_P)	5'-FAM-CGATAAACCCCGATAGACCTTACCAACCC-TAMRA-3´

Table 11: Primer and probe sequences for the detection of ruminant housekeeping gene 12S.

Primer	Sequence
Forward (12S_F)	5'-GCGGTGCTTTATAYCCTTCTAGAG-3´
Reverse (12S_R)	5'-TTAGCAAGRATTGGTGAGGTTTATC-3´
Probe (12S_P)	5'-VIC-AGCCTGTTCTATAAYCGAT-MGB-3´

2.5.2 QuantiNova internal control

The QuantiNova Pathogen +IC Kit includes an internal control for RNA or DNA, which can be used as extraction or inhibition control. The detection primers and probe for the IC RNA are included in the kit and can be multiplexed with the target (Tab. 7). After we had to switch to the QuantiNova kit, the internal control from the kit was used to control RNA extraction instead of p12S as this saved an additional RT-PCR run and could be used for all types of samples. The IC RNA was diluted 1:10 and added to the sample lysate in the first step of the nucleic acid extraction with the Viral RNA Mini Kit. The ratio is 0.1 µl per 1 µl elution volume, therefore we added 5 µl 10-fold diluted IC RNA to the AVL buffer. The volume of the buffer was adjusted to 555 µl instead of 560 µl.

2.6 Genotyping nested RT-PCR

To determine the HEV genotype and subtype of the HEV positive samples a broad reactive nested typing RT-PCR was performed (Boxman et al., 2017). The first step in this protocol is to convert the viral RNA into cDNA. This was done with the RevertAid H-minus First Strand cDNA synthesis kit (Thermo Fisher Scientific, Switzerland). The outer, inner and the sequencing primers are listed in table 12. The reaction was set up as seen in table 13. The same RNA as for the RT-qPCR was used. In 0.2 µl PCR tubes the reaction mix and nucleic acid were mixed and incubated in a Thermal Cycler for 5 min at 25°C, followed by 60 min at 42° and 5 min at 70°C to inactivate the reverse transcriptase. All HEV genotypes can be detected by the typing PCR.

Table 12: Primers for the outer and inner typing PCR and sequencing primers (Boxman et al., 2017).

Primer	Comment	Sequence
Outer Forward (ORF2_fo)	CODEHO primers	5´-AAYCARGGiTGGCGYTCiGTiGARAC
Outer Reverse (ORF2-ro)		5´-GARAAiGGRCGiGAiGGRGCiGG
Inner Forward (ORF2-fi)		5´-GAGGAGGAAGCTACCTCYGGYYTiGTiATGCTYTGYAT
Inner Reverse (ORF2-ri)		5´-GGAGAAGGAGTTGGTCGRTCYTGYTCRTGYTGRTT
Sequencing Forward (ORF2-fs)		5´-GAGGAGGAAGCTACCTC
Sequencing Reverse (ORF2-rs)		5´-GGAGAAGGAGTTGGTCG

Table 13: Reaction setup RevertAid H-minus First Strand cDNA synthesis kit.

Reagent	Volume (µl)	Concentration
5x buffer	4	1x
10mM dNTP mix	2	0.5 mM
Reverse outer primer (HEV-orf2-ro-ch) 100uM	1	5 uM
RiboLock RNase Inhibitor	1	1 U/ul
RevertAid M-MuLV RT (200U/ul)	1	10 U/ul
RNA	11	Variable
Total volume	**20**	

The resulting cDNA was directly used in the first, outer PCR. In this step the HotStarTaq DNA Polymerase (Qiagen, Switzerland) was employed in the following reaction setup and cycling conditions (Tab. 14 and 15):

Table 14: Reaction setup outer typing PCR.

Reagent	Volume (µl)	Concentration
10 x Reaction buffer	2.5	1x
dNTP mix (10mM)	0.5	0.2 mM
Forward outer primer (HEV-orf2-fo-ch) 10uM	2.5	1uM
Hotstar Taq polymerase (5u/ul)	0.25	0.05 u/ul
water	14.25	
cDNA	5	Variable
Total volume	**25**	

Table 15: Cycling conditions outer typing PCR.

°C	Duration	Cycles
95	15 min	
94	30 sec	35
42	30 sec	
72	1 min	
72	10 min	
8	99 hours	

As a last step the amplified DNA was directly used in the second, inner PCR. Again, the HotStarTaq DNA Polymerase (Qiagen, Switzerland) was used. The reaction setup and cycling conditions are listed in table 16 and 17.

Table 16: Reaction setup inner typing PCR.

Reagent	Volume (µl)	Concentration
10 x Reaction mix	5	1x
dNTP mix (10mM)	1	0.2mM
Forward inner primer (HEV-orf2-fi-ch) 10uM	4	0.8uM
Reverse inner primer (HEV-orf2-ri-ch) 10uM	4	0.8uM
Hotstar Taq polymerase (5u/ul)	0.5	0.05u/ul
water	34.5	
Outer PCR DNA	1	Variable
Total volume	**50**	

Table 17: Cycling conditions inner typing PCR.

°C	Duration	Cycles
95	15 min	
94	30 sec	35
60	30 sec	
72	1 min	
72	10 min	
8	99 hours	

Of this second PCR product, 5 µl were mixed with 1 µl loading dye and run on a 1.5 % agarose-gel, the expected size was 566 bp. If there was a clear, single band the rest of the PCR product (45 µl) was purified using QIAquick PCR Purification Kit (Qiagen, Switzerland). The kit was used according to the manufacturer's instructions and DNA was eluted with 30 µl of the elution buffer included in the kit. If there were various bands with different sizes seen on the gel, a second gel was run with the remaining 45 µl of the PCR product and the band at 566 bp-size was excised. The QIAquick Gel Extraction Kit (Qiagen, Switzerland) was performed to extract the desired DNA from the gel. The DNA concentration of the sample was determined on the NanoDrop system (Thermo Fisher Scientific, Switzerland). A forward and a reverse sequencing primer which bind to the tag in primer used for the second, inner PCR were added to a tube each and the required amount of the purified DNA equally. The samples were then sent to Microsynth (Switzerland) to perform Sanger Sequencing. The expected sequence was 493 nucleotides long and part of the conserved ORF2 of the HEV genome.

2.7 HEV typing tool

The raw forward and reverse ABI files were aligned and assembled into a consensus sequence using the BioEdit 7.2 program (https://bioedit.software.informer.com/7.2/). This consensus sequence of the nested typing PCR was submitted to the publicly available, HEV typing tool (https://www.rivm.nl/mpf/typingtool/hev/) to determine the genotype and, if possible, the subtype of the virus. The sequences were submitted to the typing tool in April 2020.

2.8 Next generation sequencing (NGS)

From each cohort of samples, e.g. wild boars from Schaffhausen, the ones with a preferably low CT-value, were chosen and submitted to NGS. The exact numbers are listed in table 18. The samples were prepared according to our in-house virome protocol. Briefly, an enrichment of the viral particles was performed, consisting of filtration and nuclease treatment. Using the QIAmp Viral RNA Mini Kit nucleic acid extraction was performed, according to the manufacturer's instructions, except for adding 6 µl of mercaptoethanol instead of the carrier RNA. As a next step reverse transcription and second strand synthesis were performed. Then DNA was amplified, purified and the nucleic acid concentration was measured on the Qubit 2.0 Fluorometer (Invitrogen – Thermo Scientific, USA). To achieve an equal concentration of the samples, 3 ng DNA of each sample were topped up to 50 µl with EB buffer. The shearing of the DNA to a fragment size of 500 bp was accomplished on the E220 Focused-ultrasonicator (Covaris, USA). Libraries were prepared using the NEBnext Ultra II DNA Library Prep Kit for Ilumina (New England Biolabs, UK), running eight cycles of amplification. The molarity of the samples was measured on an Agilent 2200 TapeStation (Agilent Technologies, USA). Paired-end sequencing was accomplished on an Ilumina NextSeq machine 500 with 2x150 bp read length for the majority of the samples. An Ilumina NovaSeq machine with a read length of 1x100 bp was used for the last run, including one wild boar liver, two sausages and four samples from the FU pig farm. Raw data were quality controlled by vendor software from Illumina and reads were mapped to an inhouse database containing publicly available full-length HEV genomes of all genotypes as well as own sequences using the SeqMan NGen software from the DNAstar Lasergene Genomic suite. The SeqManPro software was used to visualise the aligned reads, generate and download the contigs. The contigs were blasted (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest related publicly available HEV strain.

Table 18: HEV positive samples submitted to NGS.

Number of samples	Material	CT value range (RT-qPCR)
7	Wild boar liver	24.29-38.94
9	Pig faecal sample	21.5-36.0
2	Pig liver	27.6-36.9
1	Pig diaphragm	36.3
5	Meat product	29.3-35.5
3	Floor sample pig farm	33.0-35.0

2.9 Phylogenetic analysis

Phylogenetic analysis was accomplished with the MEGA 6 software. For multiple sequence alignment the MUSCLE program was used. The ORF2 sequences were included in a Maximum Likelihood Tree with 1000 bootstraps, based on the Tamura-Nei model. From each HEV-3 subtype at least one, if available two reference sequences were selected from the HEV typing tool. The full-length sequences retrieved from GenBank were shortened to the 493 nt long fragment of the ORF2 we sequenced in our study, to be able to phylogenetically compare the sequences. Four

almost complete sequences from the NGS in the present study were used to build a Neighbour-joining tree with 1000 bootstraps and the Kimura-2 parameter. Again, the reference sequences were selected with help of the HEV typing tool and retrieved from GenBank. For all the reference sequences the GenBank accession number is indicated in the phylogenetic tree. The two sequences from the coinfection in the pig9038 were separated with help of the online Bioconductor Software (https://www.bioconductor.org/packages/release/bioc/html/sangerseqR.html).

2.10 Antibody testing

All samples originating from pigs and wild boars were tested for antibodies against HEV with the PrioCHECK HEV Ab porcine ELISA Kit (Thermo Fisher Scientific, Switzerland). This indirect ELISA is suitable for porcine serum and meat juice samples, but in this study we used meat juice only. The ELISA was performed according to the manufacturer's instructions. Optical densities (OD) were read in an ELISA reader (Sunrise Tecan) at 450 nm with the reference filter set at 620 nm and results interpreted as described in the manual. The samples originating from deer were tested with the Axiom HEV Ab (AXIOM Gesellschaft für Diagnostica und Biochemica GmbH, Germany) ELISA kit. This all-species ELISA is based on wells coated with recombinant HEV antigens, corresponding to structural proteins ORF2 of the virus and detects the total antibodies to HEV in human or animal serum or plasma. We used it with meat juice and all the quality control parameters looked fine. Optical densities (OD) were again read in an ELISA reader (Sunrise Tecan) at 450 nm with the reference filter set at 620 nm and results interpreted as described in the manual.

3. Results

3.1 Prevalence of HEV RNA and anti HEV-antibodies

3.1.1 Pigs

In pig livers collected at the timepoint of slaughter, only one out of 192 tested samples was virus positive. These livers were meant to go into consumption. Additionally, 105 confiscated livers not fit for consumption were tested but none of them contained HEV RNA (Tab. 19). In the three sampled slaughterhouses the seropositivity ranged from 46.6 % in Courtepin to 68.9 % in Zurich.

Table 19: Detection of HEV RNA, antibodies and sequencing results of the ORF2 in pigs from three main Swiss slaughterhouses tested in this study.

Origin	**Sample material**	**HEV RNA (RT-qPCR), no. positive/no. tested (%)**	**HEV specific Antibodies (ELISA), no. positive/no. tested (%)**	**Sequencing result ORF2, no. positive/no. tested (Subtype)**
Zurich	Liver	0/74		
	Meat juice		51/74 (68.9)	
Basel	Liver	0/58		
	Meat juice		35/58 (60.3)	
Courtepin	Liver	1/60 (0.6)		1/1 (3s(p))
	Meat juice		28/60 (46.6)	
	Confiscated livers	0/105		
Total		1/297 (0.3)	114/192 (59.4)	1/1 (3s(p))

In the mainly younger pigs sampled at two carcass collection points in Lucerne the first sample material to be examined was the liver. Seven out of 54 liver samples contained viral RNA and 27.7 % of the animals were seropositive. The seven positive animals were examined more closely and the corresponding faecal and diaphragm sample of each pig was tested on HEV. The faecal sample resulted in the lowest Ct-values in comparison to the other material in all the pigs. The diaphragm sample was tested positive only in four out of seven pigs and with the highest CT-values. Three out of this group of virus positive pigs had already seroconverted (Fig. 4).

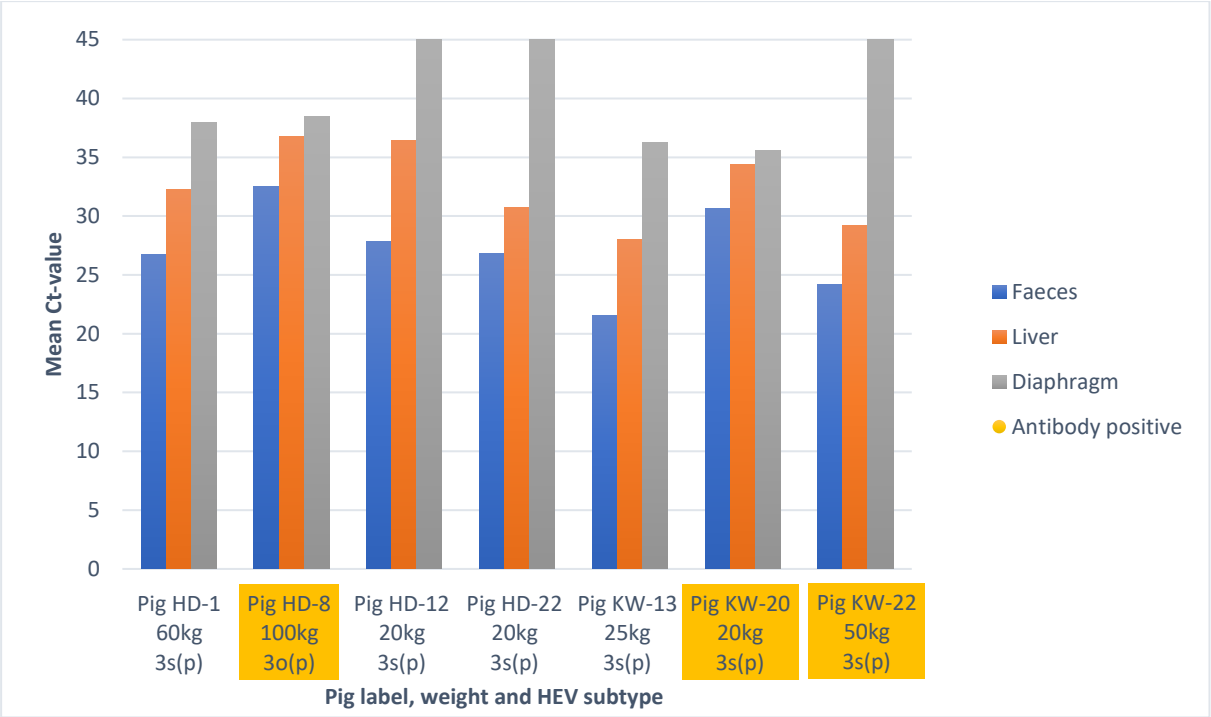

Figure 4: Comparison of mean CT-values between different sample types of the seven HEV-RNA positive animals from the carcass collection points. A CT value of 45 indicates that the corresponding sample type from this pig was negative in the RT-qPCR. Every sample was submitted to RT-qPCR twice to confirm the positive result and out of these two CT-values the mean CT was calculated.

Of the 70 samples from the FU pig farm 13 turned out to be positive in the RT-qPCR screening. The details of the positive ones are presented in table 20. In total six out of eight floor samples and three out of seven slurry samples contained viral RNA. The dust samples did not give any positive results.

Table 20: Overview of the environmental and single animal samples from the FU-pig farm. Three single animal faecal samples (one from timepoint K1 and two from timepoint K3) could not be assigned to a pig pen because of missing information. The four-digit number indicated in the last row in clamps is the individual pig number, which will be used subsequently to identify the HEV-positive pigs. The total number shows how many pigs were housed in this certain pig pen. Out of these only a certain amount of pigs was sampled individually and could be tested on HEV (Row "Number tested"). + =HEV positive environmental sample, - =HEV negative environmental sample.

Pig pen (PP)	**Timepoint and calendar date of sampling**	**Environmental samples**			**Faecal samples single animals**		
		Floor swab	*Liquid manure*	*Dust swab*	*Total number*	*Number tested*	*Positive (Pig no)*
1	K1, 03-08-2017	+	+	-	22-30	2	0
2	K1, 13-09-2017	+	-	-	22-30	19	1 (9038)
3	K1, 17-10-2017	-	-	-	22-30	7	0
4	K3, 13-09-2017	+	-	-	~30	3	0
5	K3, 17-10-2017	+	-	-	~30	6	2 (8991, 9299)
6	K3, 17-11-2017	+	+	-	~30	6	1 (7305)
7	K3, 14-12-2017	+	+	-	~30	3	0

Of all four positive animals two samples were available, one taken at the age of approximately ten weeks (K1) and one taken at day 14-21 of the fattening period (K3). The animal 9038 was tested virus-positive at the first sampling timepoint, but negative

on the second. Two pigs, numbers 8991 and 9299, show the exact opposite result. The K1 sample of the animal 7305 tested indeterminate (one positive and one negative RT-qPCR result). On the second sampling timepoint it was positive. No pig was positive on both timepoints (Fig. 5). In all the pig pens where one of these pigs was housed, the corresponding floor swab taken on the same day was HEV-positive as well. In the case of pig 7305 even two of the corresponding environmental samples, the floor swab and the liquid manure sample, were positive.

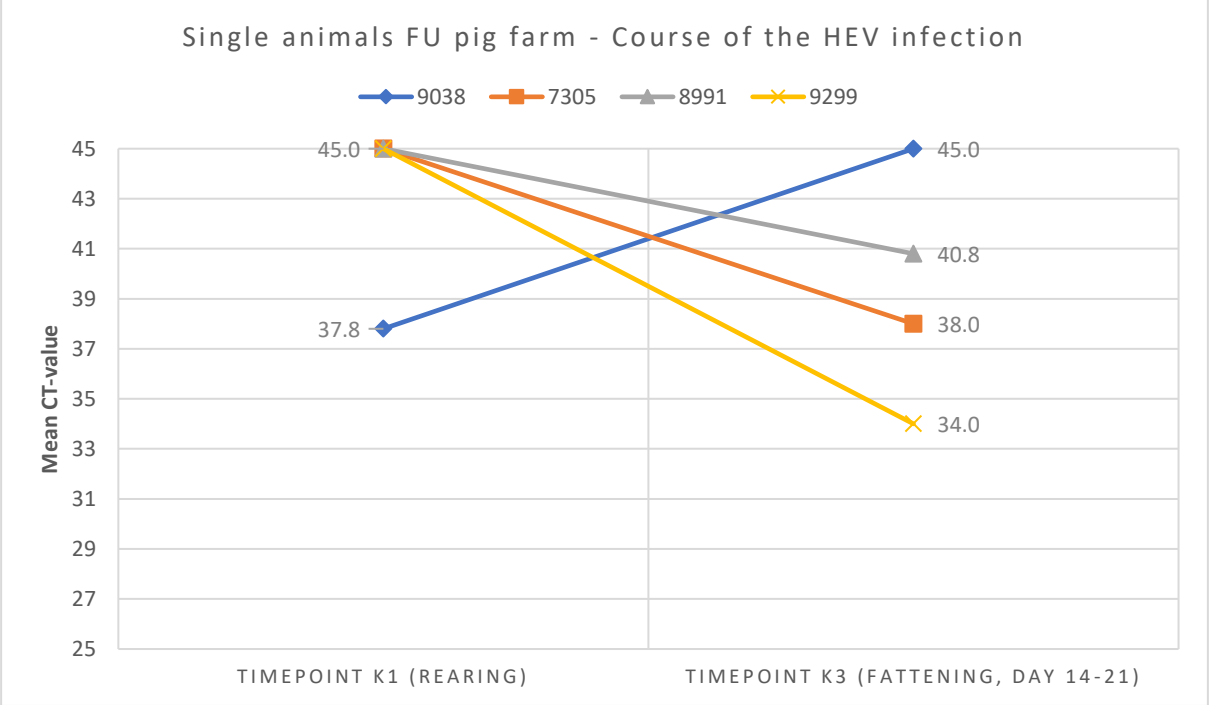

Figure 5: Course of the HEV infection in four virus positive pigs from the FU-farm. If the CT-value is indicated with 45 the corresponding sample was negative at that timepoint.

3.1.1.1 Porcine 12S and internal control of the QuantiNova kit

In the pig samples the porcine 12S was used as housekeeping gene to ensure a sufficient RNA-content for the RT-qPCR screening. When we switched to the QuantiNova kit, the internal control of the kit was used as extraction control. The CT-values of the p12S or the internal control of the QuantiNova kit are shown in table 21.

Table 21: Range of the porcine 12S and the QuantiNova Internal Control CT values for the different sample types.

Sample material	Porcine 12S	Internal control QuantiNova kit
Pig liver	6-20 12-26 (Diluted 100-fold)	
Pig faeces	27-34 (Diluted 100-fold)	19-23
Pig diaphragms	12-25	
Floor swabs	20-24	
Dust swabs		31-34
Liquid manure	26-28	

3.1.2 Wild boars

HEV RNA could be detected in seven out of 75 tested liver samples from hunted wild boars in the canton Schaffhausen. The seven samples were subtyped and five viruses belonged to the proposed subtype 3s, one to the subtype 3o(p). In total 592 samples were examined for antibodies and we received an overall seropositivity of 12.3 %. The detailed information is listed in table 22. If more than one animal of a group shot on the same date in the same region was seropositive, we also checked for viral RNA in these diaphragm samples. However, no diaphragm from these groups of wild boars was tested positive for viral RNA.

Table 22: Detection of HEV RNA, antibodies and sequencing results of the ORF2 in wild boars tested in this study.

Origin	**Sample material**	**HEV RNA (RT-qPCR), no. positive/no. tested (%)**	**HEV specific Antibodies (ELISA), no. positive/no. tested (%)**	**Sequencing result ORF2, no. positive/no. tested (Subtype)**
Schaffhausen	Liver Diaphragm	7/75 (9.3) 0/3	21/75 (28) 23/141 (16.3)	6/7 (5x3s(p), 1x3o(p))
Aargau	Diaphragm		4/87 (4.6)	
Zurich		0/12	10/64 (15.6)	
Unknown (AG or ZH)			1/55 (1.8)	
Basel-Land		0/3	7/92 (7.6)	
Solothurn			0/6	
France			1/10 (10)	
Germany			1/6 (16.6)	
Czech Republic			2/6 (33.3)	
Ticino	Liver	0/46	3/46 (6.5)	
Austria		0/4	0/4	
Total		7/143 (4.9)	73/592 (12.3)	6/7 (5x3s(p), 1x3o(p))

In the case of Schaffhausen information on the approximate age of the hunted animals was available and therefore seropositivity per age group could be investigated (Fig. 6). The group of the juveniles contains the largest number of virus positive animals and the percentage of antibody-positive wild boars was highest. For seven animals no information on the age was available, one of them being virus- and one virus- and antibody-positive. In total 43 female wild boars were tested, resulting in 12 seropositive animals. The male-group is represented with 25 individuals in total and seven antibody positive boars. For the rest of the wild boars from Schaffhausen (n=7) the information on the sex was missing.

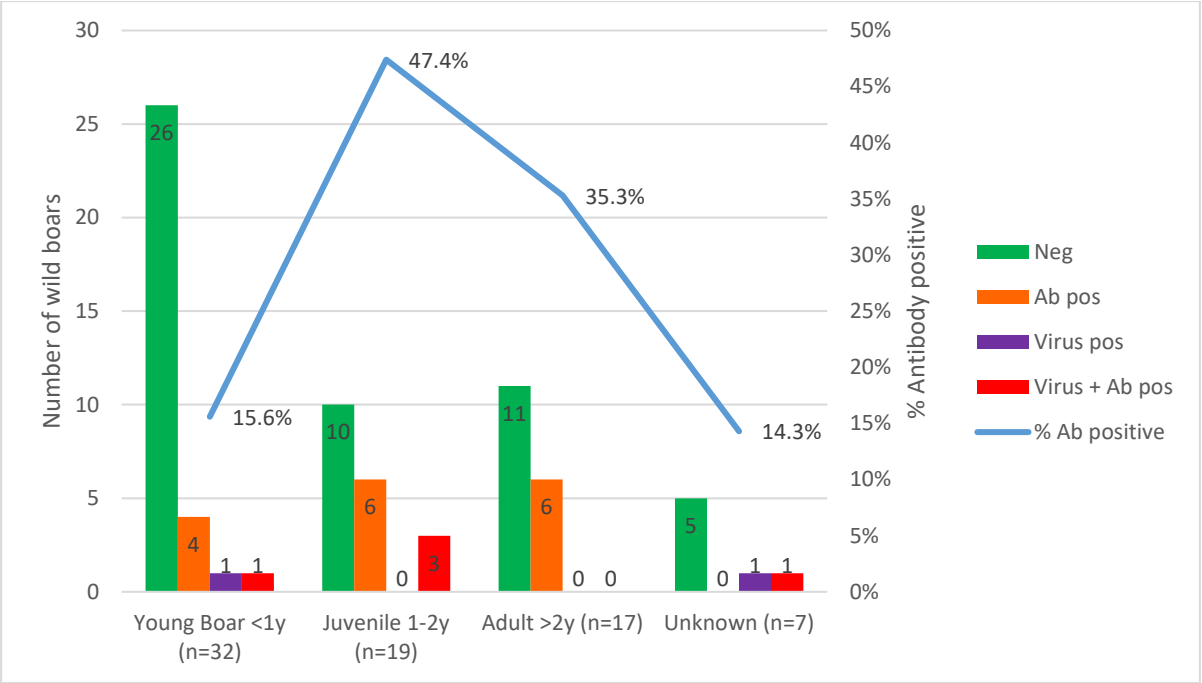

Figure 6: Number and prevalence of wild boars from Schaffhausen with anti-HEV-antibodies and viral RNA by age group. Ab=Antibody.

In figure 7 one can see the distribution of the diaphragm samples in the cantons Zurich, Aargau, Schaffhausen, Basel-Land and Solothurn from the wild boars.

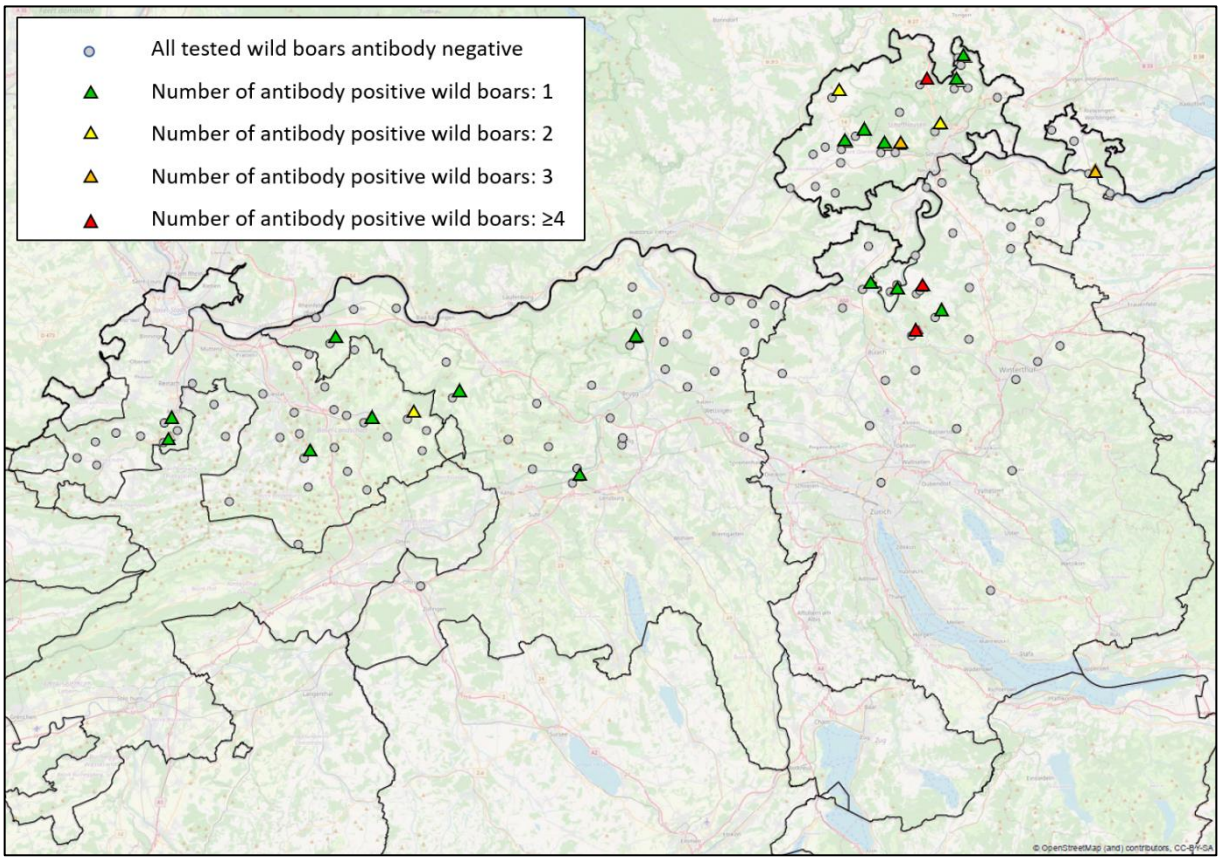

Figure 7: Distribution of the antibody positive and negative diaphragm samples from the wild boars in the cantons Schaffhausen, Aargau, Zurich, Basel-Land and Solothurn.

More detailed information was available for the canton Schaffhausen per hunting ground. The first map on top in figure 8 shows in which hunting grounds how many virus positive animals were shot and the second the same information for the antibody positive animals.

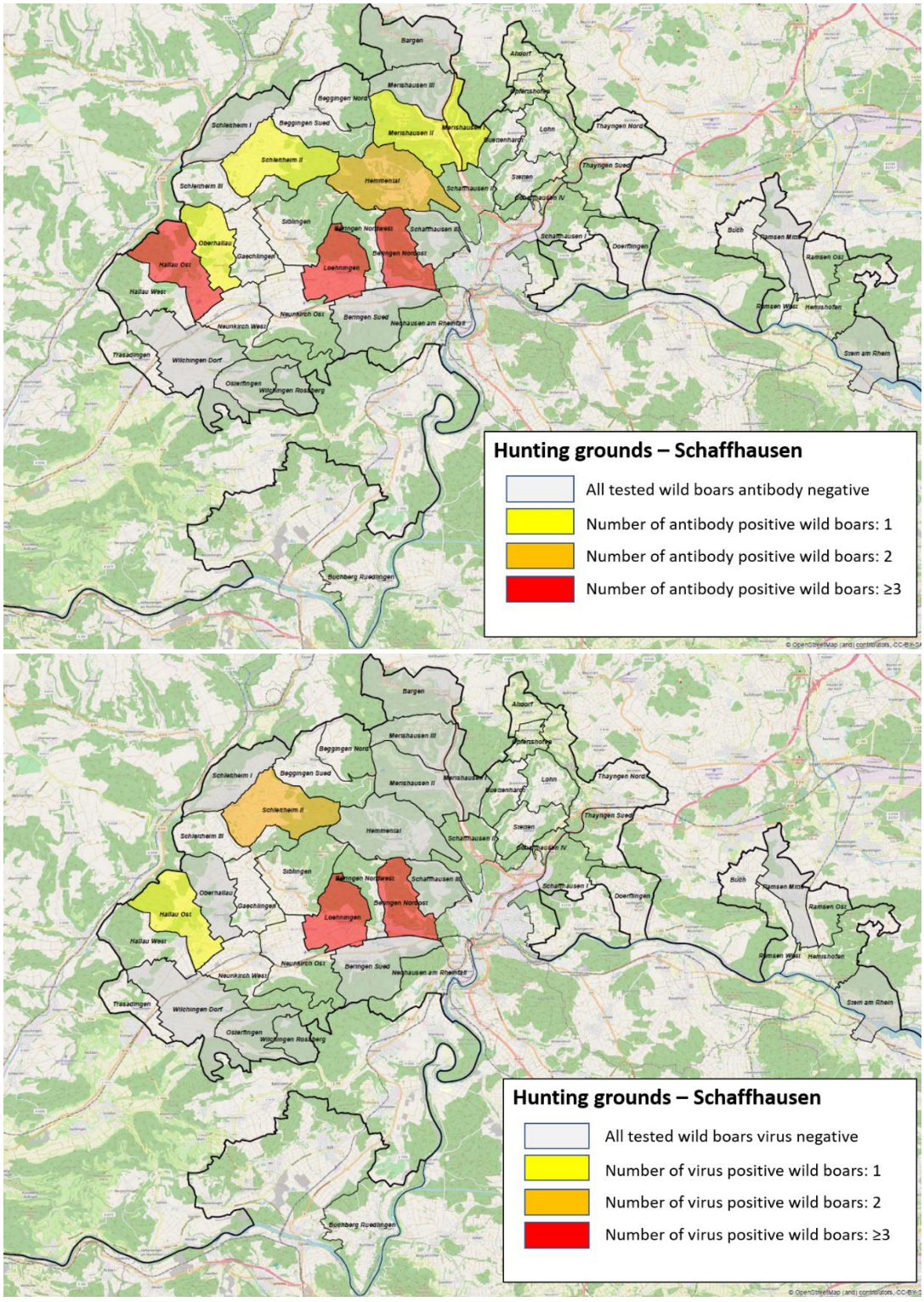

Figure 8: Detailed maps showing the distribution of the virus (top) and antibody (bottom) positive wild boar samples in the canton Schaffhausen. From the hunting grounds coloured in grey animals were tested but no wild boar tested positive.

3.1.2.1 Internal control: Porcine 12S and internal control of the QuantiNova kit

The porcine 12S encoding-gene was used as extraction control also in the wild boar samples. In the wild boar livers, the CT-values for the undiluted porcine 12S varied between five up to 20 in some single animals. Due to the often very low CT values we started to dilute the input RNA for p12S 100-fold with PBS before the RT-qPCR. Diluted, the values varied between 14 up to 31. In the few wild boar diaphragms tested in the RT-qPCR we used the QuantiNova kit with the included internal control to ensure that the extraction worked well.

3.1.3 Deer

The roe deer liver samples from the canton Schaffhausen were all HEV negative. One muscle tissue sample from Germany was seropositive (Tab. 23).

Table 23: Detection of HEV RNA and antibodies in roe deer tested in this study.

Origin	Sample material	HEV RNA (RT-qPCR), no. positive/no. tested (%)	HEV specific Antibodies (ELISA), no. positive/no. tested (%)
Schaffhausen	Liver	0/14	0/14
Germany	Muscle tissue	0/1	1/1 (100)
Total		0/15	1/15 (6.6)

The RNA-extraction was ensured by the bovine 12S for the roe deer samples. The CT-values varied between 21 up to 35 for the bovine 12S.

3.1.4 Meat products

Out of the 21 HEV positive samples from the BLV we managed to subtype nine and out of the 11 received from the Ticino six. The majority of these were assigned to the proposed subtype 3s, except for two samples which originated from Germany and were assigned to subtype 3c. In none of the samples from Bell AG viral RNA could be detected. One privately acquired sausage was weak positive in the RT-qPCR but sequencing was not feasible (Tab. 24). All the positive food samples are listed with details in table 25. Most of the HEV-positive meat products are specialities like *Mortadella*, *Salsiz* or *Saucisson*.

Table 24: Detection of HEV RNA and subtyping sequencing results of the ORF2 from meat products tested in this study.

Origin	Comment	HEV RNA (RT-qPCR), no. positive/no. tested (%)	Sequencing result ORF2. No. positive/no. tested (Subtype)
BLV Berne	21 confirmed HEV RNA-pos. samples	Not tested	9/21 (7x3s(p), 2x3c)
Cantonal Laboratory TI	11 confirmed HEV RNA-pos. samples	Not tested	6/11 (3s(p))
Bell Food Group		0/20	
Privately acquired		1/13 (7.7)	0/1
Total		1/33 (3)	15/33 (13x3s(p), 2x3c)

Table 25: Detailed information on HEV-RNA positive meat products. Every sample was submitted to the RT-qPCR twice to confirm the positive result; out of these two CT-values the mean was calculated.

Sample	Material	Origin	Mean CT-value RT-qPCR	Genotype and subtype ORF2
BLV 00672	Bauernhofleberwurst	Rügenwalder Mühle Deutschland	28	3c
BLV 00691	feine Gutsleberwurst Pommersche		27	3c
BLV 00735	Lebersalsiz	Strub Metzg Splügen	38	3s
BLV00754	Mortadella cruda	Rapelli SA	32	3s
BLV00761	Mortadellad cruda	Salumeria Malvaglia	34	3s
BLV00916	Leberwurst	Metzgerei Pally	29	3s
BLV01189	Saucisse aux choux Vaudoise	Migros Midi-Coindet	33	3s
BLV01185	Saucisse aux choux Vaudoise	Manor Food Vevey	32	3s
BLV01126	Hirschsalsiz	Metzgerei Hefti	37	3s
16LA03650	Mortadella cruda	Ticino	34	3s
16LA05705	Mortadella di fegato		31	3s
16LA06807	Mortadella di fegato		30	3s
17LA00702	Mortadella di fegato cruda Rosetta		24	3s
17LA00703	Mortadella di fegato cruda lunga		29	3s
17LA01811	Mortadella di fegato		36	3s
FO4	Figatellu	France	36	Not detected

3.2 Subtyping

The subtyping PCR was performed on 26 samples of animal material and on 15 different meat products. All subtyped samples originating from Switzerland belonged either to the proposed subtype 3s or to the proposed subtype 3o. As mentioned above, two food samples from Germany were assigned to the subtype 3c. In one case the subtype could not be assigned by the HEV typing tool, but the virus clearly belonged to genotype 3. A single animal faecal sample from the FU pig farm was coinfected by the subtypes 3s(p) and 3o(p) (Tab. 26).

Table 26: Details of all subtyping-positive samples from this study, except for the meat products which are not included here (Tab. 25). Every sample was submitted to the RT-qPCR twice to confirm the positive result; out of these two CT-values the mean was calculated. CCP=Carcass collection point, PP=Pig pen.

Sample	Details	Mean CT value RT-qPCR	Subtype ORF2
WB12	Wild boar liver, Hallau Ost SH, female, 20kg	37.45	3o(p)
WB33	Wild boar liver, Beringen NO-Löhningen SH	27.63	3s(p)
WB36	Wild boar liver, Beringen NO-Löhningen SH, female 20kg, younger than 1 year	30.93	3s(p)
WB39	Wild boar liver, Beringen NO-Löhningen SH	33.17	3s(p)
WB40	Wild boar liver, Schleitheim 2 SH, female, 28kg, 15 months	33.65	3s(p)
WB74	Wild boar liver, Schleitheim 2 SH, male, 55kg, 14 months	33.35	3s(p)
PL148	Pig liver, slaughterhouse Courtepin	37.30	3s(p)
PL-HD1	Pig liver CCP Hochdorf, 60kg	32.23	3s(p)
PL-HD8	Pig liver CCP Hochdorf, 100kg	36.80	3o(p)
PL-HD12	Pig liver CCP Hochdorf, 20kg	36.43	3s(p)
PL-HD22	Pig liver CCP Hochdorf, 20kg	30.74	3s(p)
PL-KW13	Pig liver CCP Knutwil, 25kg	27.99	3s(p)
PL-KW20	Pig liver, CCP Knutwil, 20kg	34.38	3s(p)
PL-KW22	Pig liver, CCP Knutwil, 50kg	29.24	3s(p)
PP1			
Floor swab	Pig farm FU, K1	36.04	3o(p)
Liquid manure	Pig farm FU, K1	40.47	Gt3 subtype unassigned
PP2			
Floor swab	Pig farm FU, K1	35.34	3o(p)
9038	Pig farm FU, single animal 9038, faecal sample K1	37.82	Coinfection 3o(p)/3s(p)
PP4			
Floor swab	Pig farm FU, K3	33.78	3o(p)
PP5			
Floor swab	Pig farm FU, K3	38.43	3o(p)
9299	Pig farm FU, single animal 9299, faecal sample K3	33.95	3o(p)
PP6			
Floor swab	Pig farm FU, K3	35.19	3o(p)
Liquid manure	Pig farm FU, K3	39.85	3o(p)
7305	Pig farm FU, single animal 7305, faecal sample K3	37.99	3o(p)
PP7			
Floor swab	Pig farm FU, K3	34.26	3o(p)
Liquid manure	Pig farm FU, K3	38.73	3o(p)

3.3 NGS

NGS for whole genome sequencing was performed with samples with a relatively low CT-value, ideally below 30, rising the chances to get a good genome coverage. Results range from 100 % genome coverage, meaning full-genome sequencing, to no detection of HEV reads at all. Good sequencing results could be gained from the wild boar livers. In this group three virus genomes were sequenced almost entirely. Also, the pig faecal samples from the carcass collection points resulted often in a good genome coverage. Detailed results are shown in table 27, which includes the CT-value and the percentage of the HEV genome which could be covered by NGS. In figures 9

and 10 all 3s(p) and 3o(p) sequences, respectively, from this study have been aligned against a reference sequence from NCBI GenBank.

Table 27: Details and results of all samples analysed by NGS in this study. Every sample was submitted to the RT-qPCR twice to confirm the positive result and out of these two CT-values the mean was calculated.

Sample	Mean CT value RT-qPCR	Material	% of the HEV genome covered
WB12	36,58	Wild boar liver	12.5
WB31	38,94		3.6
WB33	24,29		99.1
WB36	28,99		99
WB39	33		60.7
WB40	33.6		100
WB74	33		56.9
PF-HD1	26.7	Pig faeces	88
PF-HD8	32.5	Pig faeces	No reads
PF-HD12	27.9	Pig faeces	69.1
PF-HD22	27.0	Pig faeces	80.8
PL-KW13	28	Pig liver	88.6
PF-KW13	21.5	Pig faeces	98
PD-KW13	36.3	Pig diaphragm	No reads
PF-KW20	30.7	Pig faeces	No reads
PF-KW22	24.2	Pig faeces	75.5
PL148	36.9	Pig liver	No reads
BLV0672	35.5	Liver sausage	No reads
BLV0691	29.3	Pomm. Gutsleberwurst	No reads
BLV0735	35.4	Liver Salsiz	No reads
BLV01189	33	Saucisse aux choux	1.1
BLV01185	32	Saucisse aux choux	1.4
PP2-pig9038	37.5	Faecal sample, pig 9038	1.3
PP5-pig9299	34	Faecal sample, pig 9038	4.8
PP1-Floor swab	35	Floor swab pig farm FU	No reads
PP4-Floor swab	33	Floor swab pig farm FU	10.7
PP7-Floor swab	34	Floor swab pig farm FU	24.4

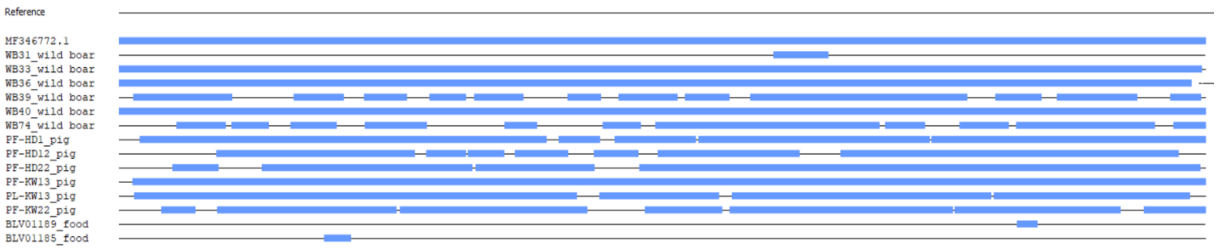

Figure 9: Alignment of all the 3s(p) NGS sequences against the reference sequence MF346772.

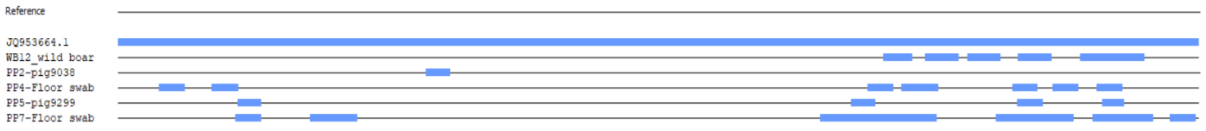

Figure 10: Alignment of all the 3o(p) NGS sequences against the reference sequence JQ953664.

3.4 Phylogenetic analysis

In total 42 ORF2 sequences from this study were phylogenetically analysed in a Maximum Likelihood tree using MEGA6. All sequences originating from Switzerland cluster within the proposed subtypes 3s or 3o. Two sequences originating from

Germany belong to the subgroup 3c. All the different types of samples from the FU pig farm cluster within the same subgroup in the 3o(p) subtype, except for the single animal faecal sample 9038. In the case of the single animal 9038 with the coinfection of two subtypes, we included both, the major 3s(p)-sequence and the minor 3o(p)-sequence in the tree. One wild boar and one pig from the CCP Hochdorf are also classified in the 3o(p)-group. The rest of the pig samples and the food samples mix in the 3s(p) cluster (Fig. 11). Four (almost) complete HEV genomes, three wild boars and one pig from a carcass collection point, were included in a full genome length Neighbour-Joining tree (Fig. 12). Only 0.9 % (WB33), 1 % (WB36) and 2 % (PF-KW13) of the genome was missing in these samples, in the case of WB40 the whole HEV genome was sequenced. The classification of the sequences stays the same, whether the ORF2 sequence or the full-length genome is used.

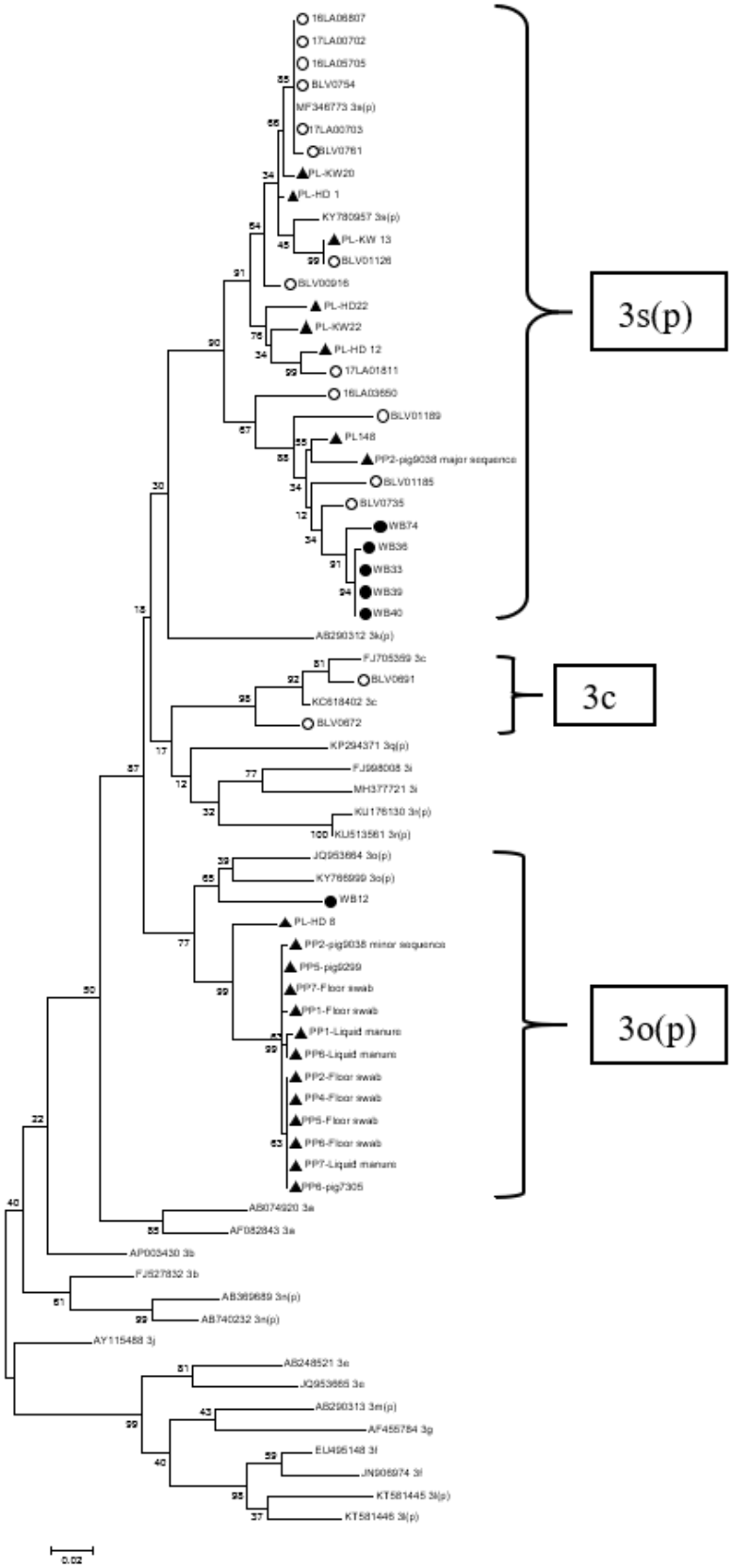

Figure 11: Phylogenetic analysis of 42 sequences from the HEV ORF2 (493nt) with the Maximum Likelihood Method and 1000 bootstraps in the Tamura-Nei model, aligned with MUSCLE. All samples from the present study are marked with the following symbols: ○food ▲pigs ●wild boars. The rest of the included sequences are reference sequences from NCBI GenBank. Only genotype HEV-3 sequences are included since all identified sequences belong to this genotype.

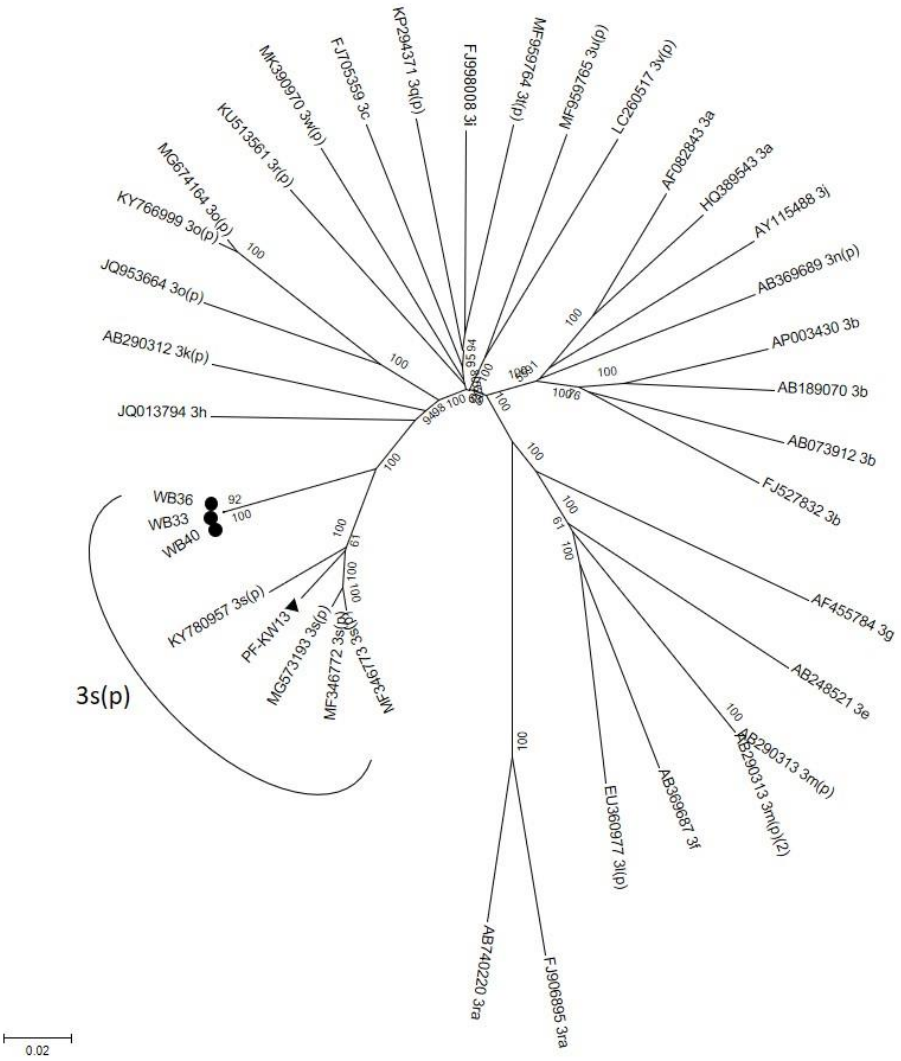

Figure 12: Neighbour-joining tree with 1000 bootstraps and the Kimura-2 parameter including four (almost) full-length HEV-sequences from this study. Only full-length HEV reference sequences from genotype 3 are included. All samples from the present study are marked with the following symbols: ▲pigs ●wild boars. The rest of the included sequences are reference sequences from NCBI GenBank. Only genotype HEV-3 sequences are included since all identified sequences belong to this genotype.

4. Discussion

In this study, we tested different reservoir hosts and meat products for Hepatitis E virus. If a sample was positive it was further submitted to a subtyping PCR and in some selected cases to NGS for further sequencing.

4.1 Pigs – Prevalence and genetic diversity

The fattening pigs at slaughtering age (app. 6 months old) that we tested for HEV had a seropositivity of 59.4 % and only one out of 297 animals was virus positive (Tab. 19). In contrast, the mainly younger pigs sampled at the carcass collection points show the opposite tendency, as 13 % of these pigs were positive for the virus and 27.7 % had antibodies against it. The pigs from the carcass collection points were mainly younger than 6 months, according to their weight. Only one pig weighed 100 kg and was therefore surely 6 months old or even older, but the others ranged between 20-60 kg. A Japanese study shows a similar pattern of the anti-HEV-antibodies and the presence of HEV-RNA in domestic pigs. The seroprevalence rises steadily from 10 % in one month old pigs up to 86 % in four months old pigs and keeping almost this level until the age of six months (84 %). The viral RNA prevalence shows the opposite tendency, with 14 % positive animals at the age of three months and 0 % at the age of six months (Takahashi and Okamoto, 2014). A higher viral prevalence of HEV was also detected in younger pigs (3-4 months old) compared to older ones (9-10 months old) in a study from Italy (Di Bartolo et al., 2011). The RNA prevalence in the slaughterhouse, which is the most relevant for the consumer, was determined to be very low in the present study (0.3 %, as presented in table 19). Müller et al. tested 160 pig livers of 40 different Swiss fattening farms for HEV RNA and the prevalence was 1.3 % (Müller et al., 2017). In conclusion we could confirm the previously shown little numbers of HEV positive pigs at slaughtering and Switzerland seems to have a low prevalence by comparison to other European countries. To allude two of our neighbouring countries, in France Rose et al. detected HEV viral RNA in 4 % of the pigs and in Italy even 20.8 % of the animals in the slaughterhouse were tested positive (Di Bartolo et al., 2011; Rose et al., 2011). The fact that younger pigs are more often virus positive but less often antibody positive indicates, that most animals get infected when they are younger than six months (before the slaughtering age).

Interestingly, in all the HEV-positive animals from the CCPs, the faecal sample had the lowest CT-value in the RT-qPCR and the diaphragm sample the highest. In three out of seven animals positive in liver and faeces no viral RNA could be detected in the diaphragm sample. These details are presented in figure 4 of the results chapter. Concerning the consumption of pork meat this seems to be good news, as the muscular tissue may be virus negative even if the liver is positive. Faecal samples are an interesting and non-invasive way to test pigs on HEV as in the faecal sample the viral load was highest according to CT-values and all pigs tested positive in the liver tested also positive in the faecal sample. Additionally, the virus can be detected during a longer time period in the faeces compared to other sample types, like for example serum. An experimental study with pigs revealed, that the viremia lasts from 10-40

days and does not happen in all infected animals, whereas the shedding in faeces was observed in all pigs and goes on from 50-80 days (Kanai et al., 2010). At this point we have to add, that it would be interesting to know how many pigs would have been positive in the faecal sample but negative in the liver. However, in this study this was not investigated, as the RT-qPCR result for the liver tissue was decisive for the definition if a sample was positive or negative. For the transmission of the virus to humans the liver is also more relevant than the faeces.

When the question of the screening of pig farms for HEV appeared, the FU pig farm was tested as environmental and individual samples from this farm were already available. Six floor swabs from pig pens and three liquid manure samples tested positive in the RT-qPCR, additionally four faecal samples from single animals contained viral RNA. The floor swabs could be an interesting way to screen pig pens for HEV, as in all the pens with one or two HEV positive pigs the floor swab tested positive as well. However, we also found positive floor swabs in pens where no single animal was tested positive. This can be explained by the fact that 22-30 pigs are housed in one pen and not all of them were sampled individually (Tab. 20). Another hint that the floor swab could be representative for the pigs housed in this pen, is a study indicating that the indirect transmission of HEV from one pig pen to another, without co-housing the animals, was low (Andraud et al., 2013). Hence, we could draw the conclusion, that viral RNA found in a floor swab taken in a specific pen must be originating from the animals housed in there and was presumably not introduced via indirect ways from another pen. On the single animal level, three out of four pigs started shedding HEV between the first (K1) and the second (K3) sampling timepoint, as shown in figure 5. As they were approximately three months old at the second sampling timepoint, this finding is in line with studies indicating that virus shedding occurs most often at the age of three months (Salines et al., 2017). To summarize this part, the floor swab seems to be an interesting and non-invasive tool to test pig pens for HEV. More farms will be analysed in the same way in a follow-up thesis at our institute, to determine the best non-invasive sample material to screen pig herds for HEV.

Among the pigs which were virus-positive in this study only HEV genotype 3 sequences of the proposed subtypes o (n=11) and s (n=7) were detected. One sequence could not be assigned to a subtype but belonged to genotype 3 and one sequence showed evidence of a coinfection with subtype 3o(p) and 3s(p), which is shown in detail in the subchapter phylogenetic analysis of this discussion (Tab. 26). As for the pigs, the subtypes 3s(p) and 3o(p) are the dominating sequences.

We used primarily the HEV typing tool for the subtyping of the sequences. There is no clear demarcation cut-off between the different subtypes, but the more HEVnet members, like us, use the same analysis tool and provide their sequences including metadata, even better: use the same sequencing techniques and protocols (which is an aim of HEVnet as well), the better. The comparison of sequences is more feasible, typing more reliable and the researchers could analyse the data using molecular epidemiology to reveal transmission pathways and characteristics of different subtypes. As example serves the possible connection between subtype and specific

clinical manifestation, such as neurological signs, of the disease in humans, which can be investigated more detailed with help of the metadata provided by members of HEVnet. In this study we did an own phylogenetic analysis in addition to the HEVnet typing tool, to collateralise and classify our results in more detail.

4.2 Wild boars and deer – Distribution, prevalence and genetic diversity

In figure 13 one can see the distribution of the wild boar population in Switzerland. In the North Eastern part, including the cantons Aargau, Zurich and Schaffhausen these animals are observed basically anywhere. Highest densities of wild boars are found in Geneva, Solothurn, Basel-Landschaft, Aargau, Zurich, Schaffhausen and Ticino (Meier and Ryser-Degiorgis, 2018). As Figure 13 shows wild boars are rare in the mountainous parts of Switzerland, including the canton Grisons. This explains why we did not receive any samples from this canton, although the cantonal laboratory in Chur GR participated and sent us many diaphragm samples from different cantons they test on *Trichinella*. To sum this up we can state, that our wild boar samples are quantitatively not representative for the distribution of the virus in the population, but at least samples from almost all cantons where the wild boar density is high were investigated, which can also be examined in the map in figure 7.

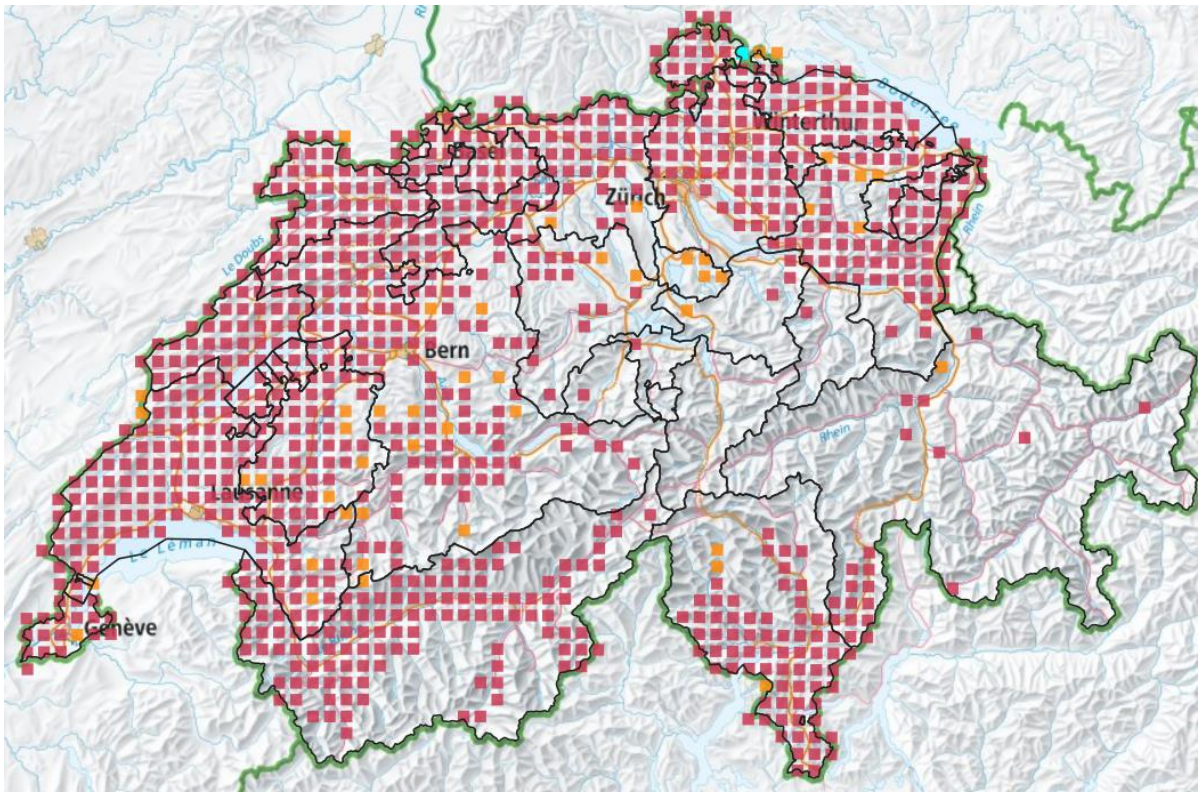

Figure 13: Distribution of wild boars in Switzerland. Pink square=observation after 2000, orange square=observation before 2000 (https://lepus.unine.ch/carto/70780).

Several animals were shot in different European countries by Swiss hunters. One particular group of these foreign samples stands out: The six wild boars shot in Stříbro, Czech Republic. In this group, two out of six wild boars are antibody-positive. It would

be very interesting to sample more animals from this area to determine if this was just coincidence or if the virus prevalence is especially high. A study from 2018 indicates that wild boars are a reservoir for HEV in the Czech Republic as well (Strakova et al., 2018).

Concerning the canton Schaffhausen more information on the individual wild boars was available. For most of the animals the weight, approximate age and the sex was provided with the samples. The group of the juveniles contained most virus-positive animals and the seropositivity reached a top level of 47.4 % in this age group. In the adult group only 35.3 % tested seropositive (Fig. 6). However, these results cannot be compared to the ones from the domestic pigs, as even the juvenile group of wild boars is already older than the fattening pig will ever be. Figure 8 reveals the large regional differences even within the small area of the canton Schaffhausen. Some hunting grounds contained several virus- and/or antibody-positive animals, others, geographically right next to it, none. We did not test a representative amount of the wild boars in each hunting ground in this study, therefore these results must be interpreted with caution. However, a study from Italy showed very similar findings from two areas very close to each other. In one area more than 30 % of the animals were virus-positive, in the other area none (Bonardi et al., 2020). Another hint for these big regional differences came up when we examined the diaphragm samples: Some batches of animals shot on the same day in the same region contained strikingly many antibody-positive animals, others none.

In 592 wild boar samples tested on anti-HEV-antibodies a seropositivity of 12.3 % resulted (Tab. 22), which is almost the same number as Burri detected in Switzerland in 2014 (Burri et al., 2014). Viral prevalence in the 75 animals from Schaffhausen was 9.3 %, this number is presented in table 22 as well. In Italy the prevalence of HEV RNA in liver samples is reported to range from 10 % (De Sabato et al., 2020) up to 31 % in some specific regions (Bonardi et al., 2020), in Germany 38 % of wild boar livers tested in some regions contained HEV RNA (Adlhoch et al., 2009). Hence, our results of the virus and antibody prevalence in the Swiss wild boar population fit in the results from the rest of Europe. In Switzerland we detected almost exactly the same amount of HEV seropositive wild boars as other authors some years ago. However, large regional differences seem to exist regarding HEV circulation in the wild boar population, as the comparably high virus- and seroprevalence results from Schaffhausen show. Maybe this could be explained by a relatively isolated circulation of a virus-strain within a sounder of boars.

Concerning the genetic diversity of the HEV sequences in the wild boars from Schaffhausen, we detected six 3s(p) and one 3o(p) strain (Tab. 26). The wild boar carrying the 3o(p)-strain was shot in the hunting ground Hallau East, which is bordering Germany. As Adlhoch et al. showed, the subtype may differ from one sounder of wild boars to the next, even if they are located very close (Adlhoch et al., 2009). To our knowledge, 3s(p) and 3o(p) have not been detected in wild boars before.

The deer samples were only a small side project in this study. As we found relatively many HEV positive wild boars in Schaffhausen, the idea to test roe deer from the same area came up, as these animals are a potential reservoir host as well. Anheyer-Behmenburg et al. tested roe deer in two subsequent hunting seasons and detected viral RNA in five out of 78 animals (Anheyer-Behmenburg et al., 2017). In total 14 roe deer samples from Schaffhausen and one from Germany were tested and none carried the virus. However, the deer from Germany was tested positive in the antibody ELISA and therefore must have had HEV contact at some point (Tab. 23).

In conclusion, wild boars are a reservoir for HEV in Switzerland and a potential risk for transmission of the virus to humans and/or domestic pigs. Contact between domestic pigs and wild boars is reported in Switzerland in all the cantons where wild boars are present (Wu et al., 2012) and transmission of the virus between these two species has been proven already: In a study experimentally infected wild boars were kept in contact with miniature pigs and infected those with HEV (Schlosser et al., 2014). People in close contact with wild boars, namely hunters or game wardens, are at a higher risk to be infected by pathogens transmitted by these animals, including HEV (Ruiz-Fons, 2017). As the wild boar population has increased over the past years (Meier et al., 2015), more animals are hunted, leading to more contact with these animals for the hunters, and more wild boar meat is supposedly consumed, which could also lead to a higher risk of Hepatitis E transmission via this route in Switzerland. However, the epidemiology and exact circulation of HEV strains in wild boar sounders is supposedly different than in domestic pigs and needs further studies.

4.3 Meat products

Concerning the meat products, the aim of this study was to determine the HEV subtype and sequence diversity of confirmed HEV-positive samples from cantonal and governmental food hygiene laboratories. However, subtyping did turn out to be more difficult in highly processed food samples than e.g. in liver or faeces, resulting in 15 sequences out of 33 RT-qPCR positive products. These sequences were retrieved from different kind of sausages and meat products. Samples which tested weak positive in the RT-qPCR could often not be subtyped successfully. Possible reasons for this problem could be a low viral load, poor RNA quality, insufficient cell lysis in the extraction step or inhibitors the meat products might contain. All 13 3s(p) sequences originated from meat products containing Swiss meat, whereas the two 3c strains were detected in German liver sausages. The subtype 3c is one of the main genotype 3 subtypes circulating in Germany. Wenzel et al. detected this subtype in porcine livers sold at retail in Germany and in another German study investigating wastewater samples it was the most prevalent subtype, to only point out two examples (Beyer et al., 2020; Wenzel et al., 2011).

The meat products in this study were mostly so-called high-risk products concerning the transmission of HEV. *Salsiz* is a specialty from the canton Grisons, which is a dry-cured salami-style prok sausage that may contain liver (*Lebersalsiz*) (https://www.patrimoineculinaire.ch/Produkte#254), the *Saucisse aux Choux* is

produced in the French part of Switzerland and contains raw pork meat, cabbage and sometimes pork liver (https://www.patrimoineculinaire.ch/Produit/Saucisse-aux-choux-vaudoise-IGP/78). The *Mortadella di fegato* sausage from the canton Ticino has been identified as source of human HEV infection in other studies (Giannini et al., 2018; Kubacki et al., 2017). This salami-style speciality contains raw pork meat and pork liver (https://www.patrimoineculinaire.ch/Prodotto/Mortadella-di-fegato/40). The single *Figatellu* from France was tested only weak positive in the RT-qPCR and no sequence could be retrieved from it. This traditional pig liver sausage, which is typically eaten raw, is a French speciality. Due to lacking routinely applicable cell-culture system it is most often not possible, to assess the infectivity of meat products without animal experimentation. However, for the specific case of the *Figatelli* this was possible with help of a cell-culture system where infectious HEV virions have been produced (Berto et al., 2013b).

Sequences of the 493 nt long part of the ORF2 retrieved from the meat products clustered within the pig and wild boar samples from this study (Fig. 11). As the sausages mainly contain meat from Swiss animals, it seems to be the logical consequence that the viral sequences are very similar. Interestingly, we did not detect any 3o(p)-sequences in the food samples, although this subtype was present in the FU pig farm, one pig from the CCP Hochdorf and one wild boar, which proves that it is present in Switzerland next to 3s(p). Of course, we only analysed a very limited amount of food samples.

In conclusion in all meat products containing Swiss meat which were subtyped successfully, we detected subtype 3s(p)-sequences. In two sausages produced with meat from Germany, two 3c sequences were found. However, the small sample amount limits the interpretation of our results concerning meat products.

4.4 Phylogenetic analysis

4.4.1 Coinfection in a pig faecal sample with subtype 3s(p) and 3o(p)

A pig faecal sample from the FU pig farm, pig 9038, could not be assigned to a subtype by sequencing the 493 nt long part of the ORF2. However, as we investigated the sequence more in detail, the electropherograms indicated the coinfection with two subtypes, as many double peaks resulted in nucleotide ambiguities. The higher peaks show a high similarity to another 3s(p) sequence from a pig liver from a slaughterhouse in Switzerland (PL148), the lower peaks to a 3o(p) sequence from the same pig farm but another group of pigs (PP7-Floor swab) (Fig. 16). The online tool https://www.bioconductor.org/packages/release/bioc/html/sangerseqR.html enabled us to separate the two sequences. To our knowledge this is the first report of a pig with a coinfection with the proposed subtypes 3s and 3o. Coinfections with other subtypes have already been reported, for example in Brazil De Souza et al. reported a coinfection with 3c and 3f subtypes in pigs (de Souza et al., 2012).

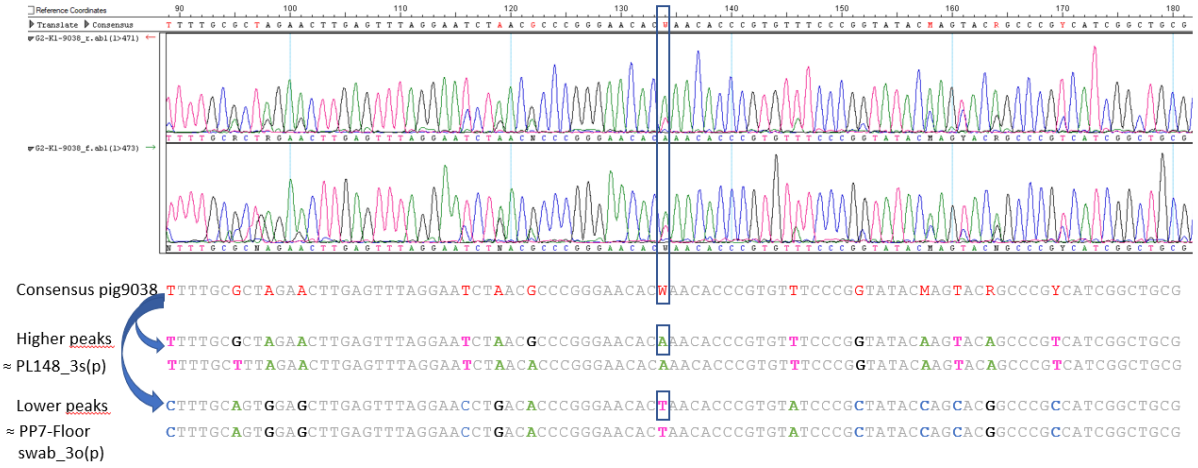

Figure 16: Visualisation of a 93 bp long part of the assembled electropherograms (reverse primer top, forward primer below) of the sequence PP2-pig9038 from an individual faecal sample. The consensus sequence below shows nucleotides with ambiguities (red). One example is highlighted with blue boxes. Considering only the higher peaks at these positions, the resulting sequence is nearly identical to a HEV 3s(p) sequence from a pig liver from the slaughterhouse while the lower peaks are identical to the HEV 3(o) sequence from another sample of this herd. This pattern is visible throughout the whole sequence.

4.4.2 Subtype 3s(p) is predominant

The sanger sequencing of the 493 nt long part in the ORF2 of the HEV-genome, which was conducted as a nested RT-PCR with CODEHO primer, worked very well in this study. This method is robust, broad reactive and feasible even with samples with relatively low viral loads (in some cases with CT value above 40). The sequenced genome part is long enough to be able to classify the virus strain into genotype and mostly also subtype. Only in two out of 41 samples the subtype could not be assigned, but one of these two was the above discussed coinfection with 3s(p) and 3o(p). The other mentioned sample was a liquid manure sample from the FU-pig farm (Tab. 26). Both samples where the subtype could not be assigned belonged clearly to genotype 3. Lu et al. proved that the ORF2 region is best suitable to determine the genotype and the subtype of HEV infections which supports our findings (Lu et al., 2006).

Out of 41 subtyped samples in this study 25 were assigned to the subtype 3s(p), 12 to subtype 3o(p), one could not be assigned (but clustered in the 3o(p)-group), two belonged to subtype 3c and one revealed a coinfection with the subtypes 3o(p) and 3s(p). In this last case both sequences, the major sequence 3s(p) and the minor 3o(p) are included in the tree (Fig. 11). In pigs, wild boars and meat products we did find the same subtypes. From humans only the few published sequences were available, especially in the case of subtype 3s(p). However, the possibility exists, that in humans other strains are circulating in Switzerland, for example due to imported meat products or as a souvenir brought back from holidays abroad. As examples serve the two 3c-sequences we detected in sausages with meat from Germany (Tab. 25). For these reasons we asked Prof. Darius Moradpour from the Centre Hospitalier Universitaire Vaudois (CHUV) in Lausanne, whether they would share some human sequences to compare to ours. Including the 31 human sequences we received, the Maximum likelihood tree displays nicely how predominant the subtype 3s(p) seems to be in

Switzerland. Additionally, the mixing, and hence the close genetic relationship, of human, pig, wild boar and food-sequences within the subtype-clusters is pictured in this phylogenetic analysis (Fig. 14).

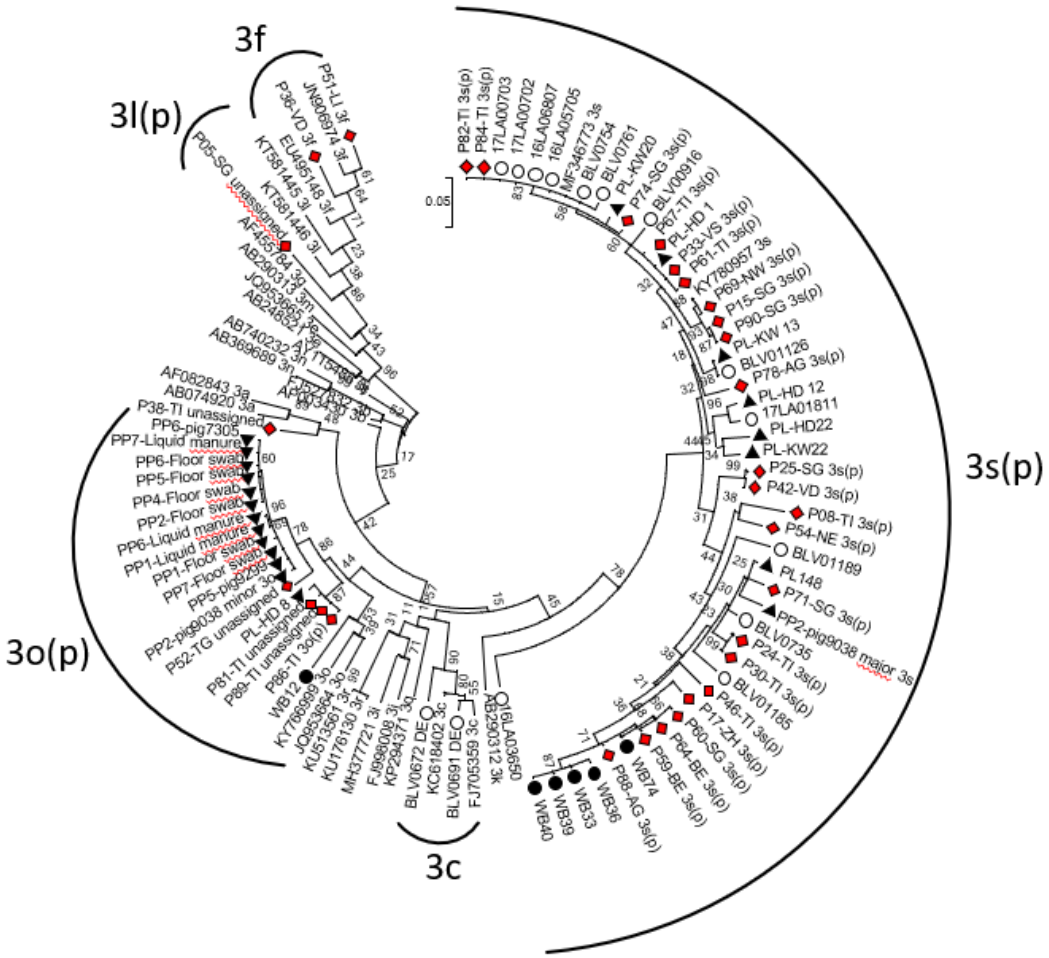

Figure 14: Phylogenetic analysis of 42 sequences from the HEV ORF2 (493nt) with the Maximum Likelihood Method and 1000 bootstraps in the Tamura-Nei model, aligned with muscle. All samples from the present study are marked with the following symbols: ○food ▲pigs ●wild boars. The 31 human sequences from Prof. Moradpour in Lausanne are marked with a red square. The rest of the included sequences are reference sequences from NCBI GenBank. Only genotype 3 HEV sequences are included, since all identified sequences belong to this genotype.

4.4.3 Subtype 3(o) – Present in Switzerland and Italy

Next to 3s(p) the second subtype detected frequently in our samples is 3o(p). According to the HEVnet database only Italy and Switzerland reported this subtype to date. The one from Italy originated from a pig faecal sample from 2012 and is closest related to our 3o(p) sequence from WB12, a wild boar shot in Schaffhausen (Fig. 15).

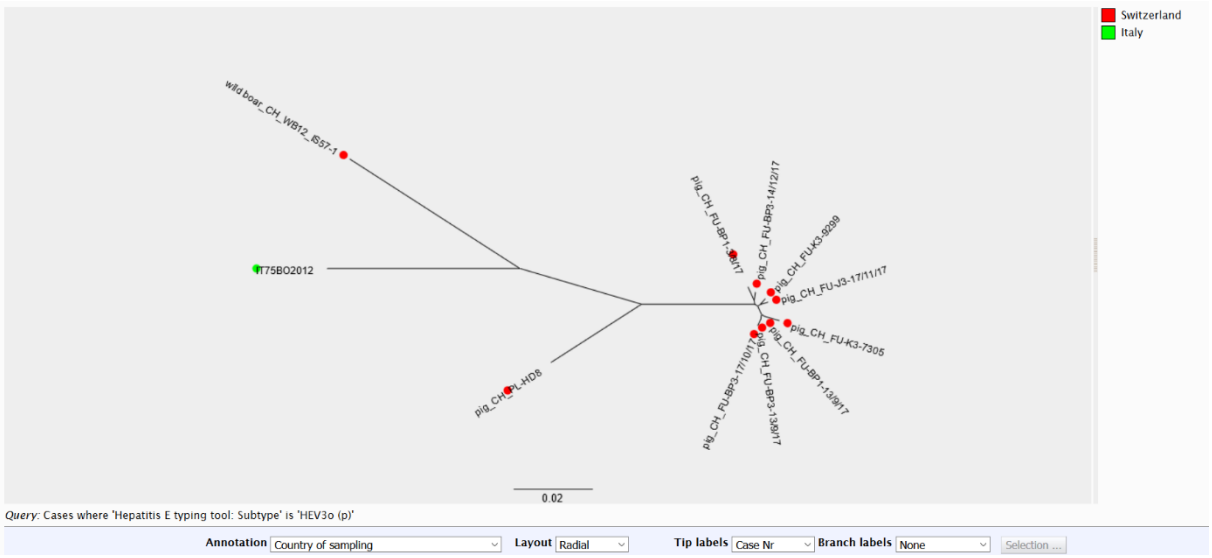

Figure 15: Screenshot from HEVnet database showing the phylogenetic tree including all 3o(p) sequences reported to the HEVnet database up to May 2020. The only sequence originating from a different country than Switzerland is displayed in green. It was sampled in 2012 from a pig in Italy.

Most 3o(p) isolates in this study originated from one single pig farm in the canton Aargau, one from a pig sampled in the CCP Hochdorf (Canton Lucerne) and one from a wild boar in Schaffhausen. Further studies on more pig farms need to elucidate the importance of this subtype in the Swiss domestic pig and wild boar population. In humans in Switzerland 3o(p) is present as well. Four of the human sequences from Lausanne cluster within the 3o(p)-group and mix with our pig sequences. Three of these human sequences even form a little subcluster together with the sequence from the pig liver PL-HD8 from the CCP Hochdorf, indicating a close genetic relationship here.

4.4.4 Switzerland – An island concerning the genetic diversity of HEV?

Although geographically enclosed by European countries Switzerland is no member of the European Union, which leads sometimes to the picture of our country being an island in Europe. Concerning the Swiss pig industry this picture is almost perfectly true. The domestic production of pork meat was as high as 95.5 % in 2018, declining a bit to 92.7 % in 2019 (https://www.proviande.ch/sites/proviande/files/2020-04/%C3%9Cbersicht_d.pdf). Also, live animals are imported very seldom into Switzerland (Personal communication Prof. X. Sidler, Swine clinics Vetsuisse Faculty Zurich). There is a quota on the number of annual imports and extensive pre-testing to proof freedom of a broad range of diseases is required. These facts facilitate the development of genetic clusters of Swiss-specific porcine viruses. A similar picture was reported in 2019 concerning the atypical porcine pestivirus (APPV) (Kaufmann et al., 2019). The authors state that all isolates analysed in their study clustered within the “Swiss subtype” of APPV, which underpins our findings and the hypothesis of a rather closed Swiss pig population.

However, regarding HEV we also have to consider the wild boar population. These wild animals will obviously cross borders without heeding the political situation and

could therefore import different viral strains into Switzerland. Especially in organic farms, where pigs can access outside areas, the contact between wild boars and domestic pigs could be close enough to transmit the virus and is absolutely not uncommon. On 17 piggeries even mating with wild boars was possible, as cross-bred piglets were born (Wu et al., 2012). A study in Corsica compared the HEV seroprevalence of two groups of pigs. One group had close contact to wild boars, the other was spatially segregated from the wild boar population. The seroprevalence in the second group, without wild boar contact, was significantly lower (Charrier et al., 2018). These findings underline the importance of the contact between pigs and wild boars concerning the transmission and/or import of HEV strains to Switzerland.

4.4.5 NGS – An insight into the viral diversity

In three wild boar liver samples and one pig faecal sample the HEV genome was sequenced (almost) entirely (Tab. 27, Fig. 12). To get deeper information of one genome or to know what other viruses are hidden in a sample, NGS is a very useful tool. As an example serves the sequencing of a liver sample from wild boar WB31 from Schaffhausen. When we sequenced this sample, different porcine viruses were detected besides of HEV, including the Torque Teno Virus, the Porcine Circovirus 3 or the Swine Pasivirus. Detecting different viruses with help of NGS, opens up new fields of research and widens our knowledge on the diversity of the viral world. This could also be particularly valuable for food products where many (potentially) zoonotic viruses may be detected in one analysis.

4.4.5.1 Challenge concerning meat products

No clear association between RT-qPCR values and genome coverage could be observed, but meat products turned out to be challenging to sequence in this study (Tab. 27). This finding contrasts earlier results in our lab, where a full-length HEV sequence from a *Mortadella*-type of sausage was published (Kubacki et al., 2017). However, this sausage had a considerably lower CT value (hence higher viral load) than the meat products sequenced in this study and was most certain to contain infectious virus, as it was linked to a human case of hepatitis E (Kubacki et al., 2017).

4.5 Limitations

One of the biggest limitations of this study was the small number of samples tested in some cohorts, like the meat products or the deer livers. Another limitation is, that our study is not representative for the respective animal population or the meat products. However, the aim of the study was to gain first information on the sequence diversity and does not claim to be comprehensive. Minor or regional subtypes may be present in Switzerland that were missed by this study. The subtyping PCR was only feasible with meat products having a relatively low CT-value, which is another limitation.

4.6 Outlook

Developing a fast, easy to sample, cost-efficient and non-invasive screening tool, for example collective faecal samples from the floor, floor swabs or manure samples, to screen the pig herds for HEV is a next aim of the HEV research in veterinary medicine

and the next doctoral thesis on this topic. The results of this study and the following one could in a next step be used to determine these three key points:

i) The epidemiological characteristics of the infection on herd level, e.g. if a geographical pattern exists or if most herds are infected or only some clusters can be detected
ii) Identify endemically infected herds and try eradicating the virus in these
iii) Build a Swiss sequence repository that enables tracing infection in humans back to the pig herd, similar to the one established for the Bovine Viral Diarrhea Virus in Switzerland (Stalder et al., 2016). This would allow analysis of chains of infections and risk factors associated with zoonotic virus transmission.

4.7 Conclusion

Pigs, wild boars and specific high-risk meat products pose a risk for autochthonous infections with HEV gt3 in Switzerland, the role of the roe deer population needs further investigation.

We can confirm, that mainly subtype 3s(p) and some 3o(p) was found in pigs, wild boars and meat products in Switzerland so far. In humans a somewhat wider genetic diversity has been observed, with subtypes 3a, 3f and 3ra but also genotype 4 sequences being present next to many 3s(p) and also some 3o(p) strains (Sahli et al., 2019). However, the provisional subtype 3s(p) seems to be the predominant subtype circulating in Switzerland. Therefore, the hypothesis of a predominant, probably Swiss-specific Hepatitis E gt3-subtype can be confirmed by our data and by the fact, that this subtype has so far only been described in Switzerland. This is supposedly linked to the isolated Swiss pig industry and the high percentage of self-supply with pork.

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